Microcalorimetric Investigations on Copper Sulfide Bioleaching

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I have not failed.
I´ve successfully discovered 10,000 things that won´t work.

Thomas Edison

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Glossary

AAS	Atomic absorbtion spectrometry
AC	Ante christum
AMD	Acid mine drainage
ARD	Acid rock drainage
ATCC	American type culture collection
CLSM	Confocal laser scanning microscopy
DSMZ	Deutsche Stammsammlung von Mikroorganismen und Zellkulturen
EPS	Extracellular polymeric substances
Gt	Gigaton
HPLC	High-performance liquid chromatorgaphy
IC	Ion-exchange chromatography
Mt	Megaton
n.a.	Not available
ORP	Oxidation/ reduction potential
Rpm	Revolutions per minutes
S^0	Elemental sulfur
SHE	Standard hydrogen electrode
USA	United States of America
UV	Ultraviolet
XRS	X-ray spectroscopy

Abstract

The aim of this work is the establishment of a microcalorimetric determination method to assign the chemical and biological degradation of copper sulfides (chalcopyrite, chalcocite and covellite). This study included the screening for microorganisms for copper sulfide bioleaching, the verification of microcalorimetric determination with different ore types and various temperatures and the thermodynamic calculation of the reaction energy $\Delta_r U$ by means of the measured heat output and the determined iron, copper and sulfate ions in the leachate. During this work microscopic observation of the microbial attachment towards chalcopyrite and the cuprous copper stability within the leachate were made.

After the adaptation of the leaching set-up during mesophilic bioleaching up to 9 % iron and 8 % copper could be recovered, during moderate thermophilic bioleaching up to 25 % iron and 12 % copper could be leached from chalcopyrite and during the bioleaching with thermophilic archaea up to 7.4 % iron and 24 % copper could be recovered. In general for every condition sterile controls leached only 25-50 % of the total iron and copper recovered by bioleaching.

Chalcocite leaching showed in the mesophilic temperature range up to 17 % copper recovery by chemical leaching and bioleaching. In contrast, with moderate thermophilic leaching only 17 % copper were leached chemically, but up to 41 % was due to bioleaching. Copper recovery during bioleaching of covellite was similar for the mesophilic and moderate thermophilic temperature ranges, i.e. 2 -15 %. During the experiments high pH values (pH > 2.5) and copper concentrations (Cu > 100 mM) were reached.

Up to 82 %, 90 % or 85 % of the recovery rates of the standard reaction energy calculated for the dissolution of chalcopyrite were calculated via microcalorimetry for mesophiles, moderate thermophiles and thermophiles, respectively. If the recovery rate of the standard reaction energy is underestimated, not all bonds within the chalcopyrite are broken and not all energy is released. Precipitation of ions (especially iron ions) could lead to an overestimation of the recovery rate.

For mesophilic chalcocite leaching 5-12 % and for moderate thermophilic leaching 32-38 % of the standard reaction energy could be detected via microcalorimetry. However, for covellite leaching 10-15 % of the standard reaction energy could be detected regardless of the temperature.

Though microcalorimetry is suitable for the assessment of bioleaching activity with chalcopyrite ores, in this study it is rather not a suitable technique for chalcocite or covellite bioleaching. However, this might be exclusively due to the used minerals themselves.

The biofilm formation on chalcopyrite was followed by the Confocal Laser microscopy. The images of microbially colonized chalcopyrite showed that the population density differed with the conditions under which the microorganisms were pre-grown. Iron sulfate and pyrite pre cultivated microorganisms showed higher attachment to chalcopyrite than chalcopyrite pre-cultivated ones. Sulfur pre grown cells were easily detachable from the mineral during the staining procedure. A high coverage of chalcopyrite by attached microorganisms does not correlate with an enhanced extraction of copper.

During the leaching experiments considerable amounts of cuprous copper could be determined within the leachate of chalcopyrite. Based on colorimetric determination of the copper speciation in solution significant amounts (up to 80 % of the total concentration) of copper were found to be present as cuprous copper.

1. Introduction

1.1 Copper mining

Copper is one of the most important raw materials in the world (Dimitrijević *et al.,* 2009). It is ranking after iron and aluminium in importance for infrastructure and technology (Sverdrup *et al.,* 2014). The annual production of copper in 2004 was 17.9 Mt/year (USGS, 2014) indicating that copper is one of the metals with the highest production rates in the world, besides iron (1200 Mt/year), aluminium (44 Mt /year), manganese (18 Mt /year), chromium (16 Mt /year) and zinc (13 Mt /year) (Sverdrup *et al.,* 2014). Copper is primarily used in end-use applications like buildings, (electrical) infrastructure, transportation and industrial equipment (Harmsen *et al.,* 2013). In Europe 58 % of the copper is used for electricity and energy purposes, followed by 26 % for building and construction, 10 % for industrial plant and machinery, furniture and coinage, and 5 % for transport (Sverdrup *et al.,* 2014). Its wide variety of uses, including electrical wiring, heat exchangers, piping and roof construction and consumer electronics makes it a valuable metal (Northey *et al.,* 2013).
Between 1988 and 2000 the world copper production rose from 8.7 Mt up to 13.2 Mt indicating that this metal is in great demand (Northey *et al.,* 2014). In 2002 the United States were overtaken by China as the world´s leading user of refined copper (Baba *et al.,* 2012). The industrialization of developing economies in Asia fuelled the demand for copper since the turn of the millennium (Dimitrijević *et al.,* 2009). India is already taking a similar path and it is expected that other regions (e.g. South America, Africa) will follow in the medium term. Therefore, it can be expected that the demand for copper will stay strong and grow for several decades (Northey *et al.,* 2014).
It is estimated that 4.89 Gt of copper are available in the world, thereof 55 % to be found in porphyry, 35 % in sediment hosted, 3 % in volcanogenic massive sulfide deposits and 7 % in other deposits (Harmsen *et al.,* 2013). There are 5 major mining areas for copper containing ores, i.e. (i) Rocky Mountains and Great Basin area in the USA, (ii) Western slopes of the Andes in Chile and Peru, (iii) Central Africa, (iv) Central Canada and Northern Michigan, and (v) areas of the former Soviet Union (Wilson, 1982). Copper is mostly present in the earth´s crust as copper-iron-sulfide and copper-sulfide minerals (Davenport *et al.,* 2002). More than 67 % of the copper production originates from porphyry copper ores. Due to the excessive extraction of copper over the last decades it is hard to find a deposit averaging more than 1 to 2 % copper (Dimitrijević *et al.,* 2009; Song *et al.,* 2011). At present, ores with a grade of 0.62 % copper in average are still mined (Northey *et al.,* 2014). Nowadays South America meets largely the demand for copper (Baba *et al.,* 2012; Northey *et al.,* 2013) with Chile and Peru having a stake of 42 % of the global copper mining. In addition, both countries also account for 38 % of the global copper reserves (Schippers *et al.,* 2014).
Copper is a limited resource on Earth (Sverdrup *et al.,* 2014) and world reserves of high-grade ores are diminishing (Mudd, 2007; Hoque & Philip, 2011). At present approximately one-third of all copper consumed worldwide is recycled (Baba *et al.,* 2012).

Copper is produced in fluid bed arrangements or flash smelters, in which the roasting, smelting and slag formation take place in a single working step. After roasting, the removal of sulfur at high temperatures as sulfur dioxide is achieved by blowing air or oxygen through the molten matte. The matte then consists out of 98 % copper (blister copper). For the refining, the blister copper is treated in anode furnaces. The deoxygenated copper is afterwards poured into moulds to produce cathodes by electro refining, with a quality of 99.99 % copper. In modern plants, 99 % of the produced sulfur dioxide is converted to sulfuric acid (an important side product of copper production) using the double contact process (Schippers *et al.,* 2014). Although today still 80 - 85 % of copper is produced through pyrometallurgical processes (concentration, roasting and smelting), much attention has been paid to hydrometallurgical processes (Brierley & Brierley, 2001; Dreisinger, 2006; Li *et al.,* 2013). Pyrometallurgy encompass several inherent constrains like high energy (22.2 GJ/t Cu in average; ca. 5 % of world´s energy production is consumed by mining operations) and capital inputs as well as high risk of secondary environmental pollution (e.g. emission of 2.6 t CO_2/t Cu; Hoque & Philip, 2011; Northey *et al.,* 2013; Johnson, 2014). Additionally, low grade ores are not always amenable to conventional floating and smelting processes (Brierley, 2008; Song *et al.,* 2011). Hydrometallurgy (or leaching as a synonym) is the chemical process used to extract minerals from ores by dissolving them in liquid (Abraitis *et al.,* 2004). Fig. 1 illustrates the copper recovery processes based on their application concerning the ore grade and type.

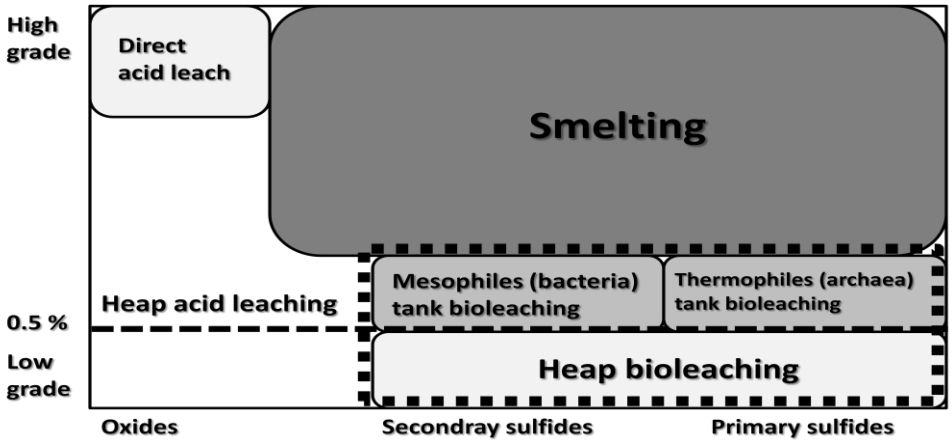

Fig. 1: Copper extraction technologies and their application based on the grade and type of the ore for processing (Anjum *et al.,* 2012)

At the beginning of the twenty-first century an increase in the share of leaching operations from 10 % to 20 % of the total copper production was reported (Schippers *et al.,* 2014).

1.2 Fundamentals of biomining

Biohydrometallurgy or biomining implies the involvement of microorganisms in the extraction of metals form ores (Hoque & Philip, 2011). It is well known that microbes participate in the deposition and solubilisation of heavy metals in the earth´s crust since geological times and that they are involved in the global iron and sulfur cycle (Gadd, 2010). As a consequence it is not astonishing that microbes can dissolve metals from minerals. The Greeks and the Romans extracted copper from mine waters more than 2000 years ago (Bosecker, 1997). The Moors carried out heap leaching at the Rio Tinto Mines during their conquest of Spain around 700 AC (Ehrlich, 2001). The dissolution of metals from ores through the action of microorganisms is called bioleaching. Nowadays bioleaching is successfully applied for industrially scale metal extraction (Brandl, 2008). In contrast to conventional mining, bioleaching has several advantages, such as moderate capital investment costs coupled with low operating costs due to the basic equipment and simple operating procedures, a lack of sulfur dioxide emission and its adaptability to the recovery of metals from low grade ores and waste materials (Watling, 2006; Brierley & Brierley, 2013).
Next to bioleaching, biooxidation plays also an important role in biomining. It is applied especially for gold and silver ores to dissolve interfering metal sulfides (e.g. arsenopyrite) bearing the precious metal (Reith *et al.,* 2007; Petersen, 2010).
Most biohydrometallurgical innovations have been commercially implemented during times of low metal prices in the 1950s with the advent of copper bioleaching at the Kennecot Copper Bingham Mine (Hoque & Philip, 2011; Anjum *et al.,* 2012). At present, Biomining is mainly utilized for the winning of copper (Watling, 2006), nickel (Watling, 2008), and the pre-treatment of refractory gold ores (Petersen, 2010). There is also a great potential for the mining of uranium (oxides) (Choi *et al.,* 2005), cobalt and zinc (Rohwerder *et al.,* 2003), and for the pre-treatment of metal ores containing rare earth metals (Hoque & Philip, 2011). Biomining of aluminium and lithium from spodumene, cobalt and nickel from laterites (du Plessis *et al.,* 2011) and cobalt, nickel, copper and manganese from nodules is currently under investigation (Schippers et al., 2014). Nowadays approximately 15 % to 20 % of the world copper, 5 % of the gold and 3 % of the nickel production are accomplished by biomining (Zechendorf, 1999; Schippers *et al.,* 2014).
There are two different ways to apply bioleaching for winning of valuable metals, the irrigation-type processes like *in-situ*, dump, heap and vat leaching, and the stirred tank process (Hoque & Philip, 2011). To optimize leaching conditions for each type of ore, there are several laboratory scale processes available such as percolators, submerged and column leaching techniques (Bosecker, 1997). *In-situ* (stope) leaching is applied in abandoned mines, with a borehole being drilled into the ore deposit and a solution with microorganisms which is pumped through the hole (Sand *et al*., 1993). This technique is applied especially for the mining of uranium in USA and Canada (Mudd, 2001). Dump leaching is the oldest and simplest technology. By this technique crushed low-grade ore or waste materials are deposited in large heaps and irrigated with iron- and sulfate rich mine wastewater. Microorganisms in the heap dissolve the metal sulfides in the ores (Schippers *et al.,* 2010).

Heap leaching is the most widely applied technique for copper mining (Donati & Sand, 2007). This is similar to dump leaching, but under better controlled environmental conditions. The low-grade ores are milled down (to centimetre sizes) in order to provide a larger surface area for the microorganisms interacting with the mineral and to allow the lixiviant to reach all the sulfide inclusions in the heap material (Ghorbani *et al.,* 2011). An impermeable sheet is placed on the bottom to collect the leachate and to avoid environmental pollution (Pradhan *et al.* 2008). This technique is most successfully applied to copper oxides and secondary copper sulfides (Watling, 2006). Vat leaching is used mainly for metal oxide leaching of tailings with high metal concentrations so far. Even though this method is very cost intensive, it is considered to be useful for the treatment of ore concentrates and precious minerals, because of the possibility of controlling the process to enhance recovery rates (Hoque & Philip, 2011). Stirred tank bioleaching is mostly applied for the pre-treatment of gold associated with arsenopyrite (La Brooy *et al.,* 2008; Gericke *et al.,* 2010). At present 80 % of the bioleached copper originates from projects with secondary copper ores (Schippers *et al.,* 2014). Process times may vary among the operations, but usually take about 200 days for leaching secondary copper ores with a recovery of 75% to 85% in most operations. Ores often contain carbonate minerals, clays or both of them resulting in a high acid consumption and may show a notable sulfur content (up to 10%) which generates a considerable heat output during their oxidation (Brierley, 2001).

1.3 Bioleaching mechanisms

Due to its industrial relevance, great research efforts to reveal the bioleaching mechanisms have been made. In 1964 Silverman and Ehrlich proposed that bacteria enhance the rate of pyrite oxidation compared to pure chemical leaching. In this context the “indirect” and the “direct” degradation mechanism were controversially discussed (Silverman, 1967). Via the direct mechanism the sulfur moiety of the metal sulfide is oxidised enzymatically, whereas via the indirect mechanism the metal sulfide is oxidised by ferric ions, which are then re-oxidised by the bacteria. Up to now there is no evidence for the direct mechanism (Sand *et al*., 2001), and the indirect mechanism is now comprised of two sub-mechanisms: the “contact” and the “non-contact” mechanism (Rawlings, 2002).
Both mechanisms postulate the oxidation of ferrous iron by bacteria. Planktonic cells leach metal sulfides via the non-contact mechanism, while the contact mechanism requires the cells to be in direct contact attached to the mineral surface (Sand *et al*., 1995). The research teams of Wolfgang Sand, Frank Crundwell and Helmut Tributsch demonstrated in the 1990´s that leaching bacteria have a strong affinity towards metal surfaces (Rawlings, 2002) and that the forming of so-called biofilms embedded in a matrix of extracellular polymeric substances (EPS, Fig. 2) enhances and promotes bioleaching (Kinzler *et al.,* 2003).

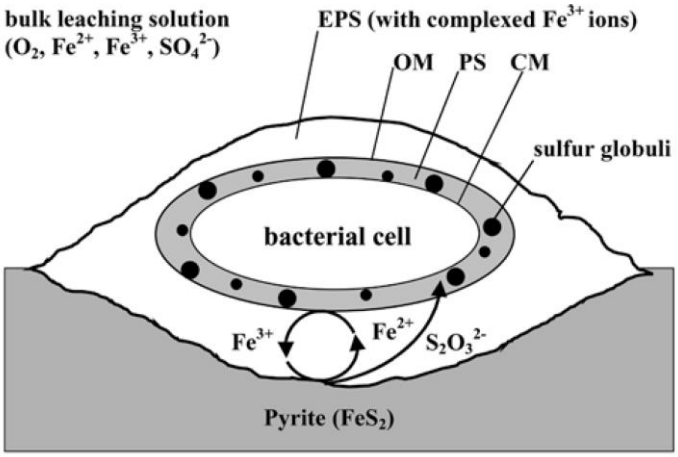

Fig. 2: Contact leaching of pyrite by cells of *Acidithiobacillus ferrooxidans* embedded in a EPS-matrix; iron(III) ions catalyze the degradation process within the EPS layer of the cell. OM= outer membrane, PS= periplasmic space; CM= cytoplasmic membrane (Rohwerder *et al.,* 2003)

In 1999 Schippers & Sand published their work about intermediates produced during dissolution of several metal sulfides. The authors observed that the dissolution process is not identical for all metal sulfides. As a result, they proposed acid-insoluble sulfide minerals such as pyrite to be leached via the thiosulfate mechanism and acid-soluble minerals such as chalcopyrite to be leached via the polysulfide mechanism (as illustrated in Fig. 3).

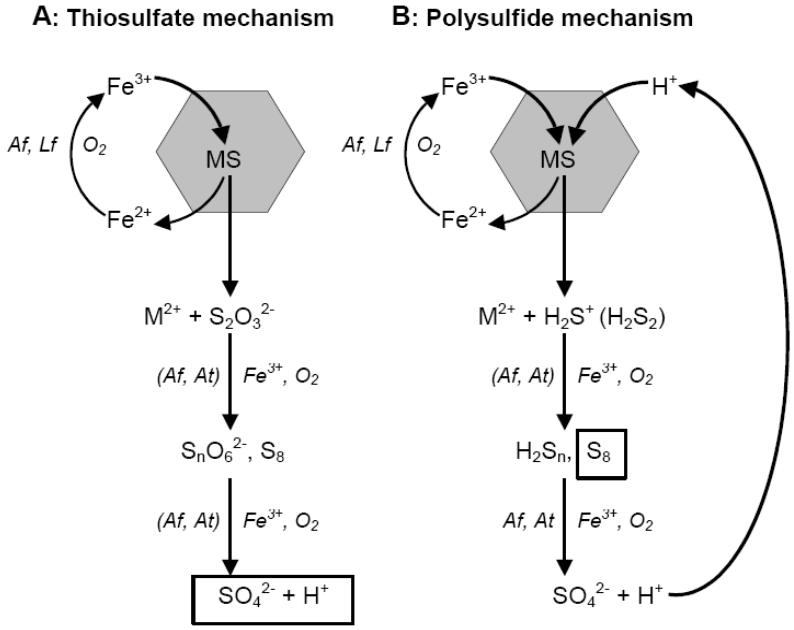

Fig. 3: Pathways of metal sulfide biodegradation after the Thiosulfate or the Polysulfide mechanism. MS= metal sulfide, M^{2+}= metal cation, Af= *Acidithiobacillus ferrooxidans*, Lf= *Leptospirillum ferrooxidans*, At= *Acidithiobacillus ferrooxidans* (Rohwerder *et al.,* 2003)

The acid-insoluble metal sulfides FeS_2 (pyrite), MoS_2 (molybdenite), and WS_2 (tungstenite) consist of pairs of sulfur atoms which form non-bonding orbitals. Consequently, the valence bands of these metal sulfides are only derived from orbitals of metal atoms, whereas the valence bands of all other metal sulfides are derived from both metal and sulfur orbitals . Thus, the valence bands do not contribute to the bonding between the metal and the sulfur

moiety of the metal sulfide leading to a high resistance against proton attack. The bonds can only be broken via multistep electron transfers (six successive one-electron oxidation steps to thiosulfate) with an oxidant like iron(III) ion. Thiosulfate is oxidized via tetrathionate and polysulphides to sulfate in chemical and/or biological reactions.
Acid-soluble metal sulfides are dissolved by the oxidation power of iron(III)ions and through proton attack. The chemical bonds between the metal and the sulfur moiety are hydrolysed by proton attack, and hydrogen sulfide is liberated. In presence of iron(III)ions the hydrogen sulfide is oxidized to a sulfide cation radical which is able to dimerize to free disulfide and higher polysulfides. The further oxidation process proceeds via polysulfides to elemental sulfur. The oxidation of elemental sulfur is exclusively carried out by microorganisms because this sulfur species is inert to abiotic oxidation in acidic environments (Schippers *et al*., 1996; Rohwerder *et al.,* 2003; Vera *et al.,* 2013).

1.4 Bioleaching microorganisms

The involvement of bacteria in metal dissolution remained undiscovered until the middle of the 20th century (Ehrlich, 2001). Microorganisms used for extraction of metal sulfides are associated in heavy metal-rich environments (Dopson *et al.,* 2003) with extremely acidic pH (Watling, 2006). There are two major origins for such acidic, metal-rich environments associated with volcanic or with mining activities, respectively (González-Toril *et al.,* 2006). Most metal-sulfide-dissolving microorganisms are able to oxidize inorganic sulfur compounds and/ or iron(II)ions and grow chemolithotrophically (Rohwerder *et al*., 2003).
In 1950 Colmer, Temple and Hinkle isolated first an iron oxidising bacterium involved in bioleaching of metal sufides from the acid mine drainages of a bituminous coal mine, *Acidithiobacillus (At.) ferrooxidans*. It is the best studied leaching bacterium, formerly known as *Thiobacillus ferrooxidans* (Kelly & Wood, 2000). It is a rod-shaped, non-spore forming, Gram negative bacterium (Harrison, 1984), which utilizes ferrous iron as electron donor and carbon dioxide as carbon source. *Acidithiobacillus* can grow aerobically on elemental sulfur or metal sulfides as well as anoxically with formate using ferric iron as electron acceptor (Pronk *et al.,* 1992). The genus *Acidithiobacillus* comprises also the species *Acidithiobacillus thiooxidans* and *Acidithiobacillus caldus*. Both are unable to utilize ferrous iron and metabolise exclusively reduced sulfur compounds (Harrison, 1982, Hallberg & Lindström, 1994). For many years *At. ferrooxidans* was considered to be the dominant microorganism in bioleaching operations at mesophilic conditions (Rawlings, 2002). Due to its higher tolerance towards acidity and ferric iron (Vásquez & Espejo, 1997) *Leptospirillum ferrooxidans* is by far more often found as the dominant iron-oxidiser. 1972 Markosyan isolated this vibrio-shaped bacterium from mine water of the Alaverda copper deposit in Armenia. Bacteria of the genus *Leptospirillum* are physiologically similar to those of the genus *Acidithiobacillus*, except they are only able to use ferrous iron as electron donor (Hippe, 2000). *Leptospirillum ferriphilum* is another representative, often found in biooxidation operations growing at moderate thermophilic conditions (Coram & Rawlings, 2002).

Sulfobacilli are Gram-positive, acidophilic, moderate thermophilic, and endospore-forming bacteria, which have been isolated from geothermal environments as well as mining operation sites (Norris et al., 1996). They can grow autotrophically on ferrous iron, sulfur or sulfide minerals, mixotrophically on ferrous iron and yeast or heterotrophically utilising glucose in a temperature range of 40 to 60°C (González-Toril *et al.,* 2006). The most prominent representative is *Sulfobacillus thermosulfidooxidans* (Watling *et al.,* 2008). Most of the mentioned bacteria are mesophiles or moderately thermophiles (Tab. 1). However, since the first thermophilic archaeon was discovered in the 1960s (Brierley & Brierley, 2013) there are now several thermophilic as well as extreme thermophilic bioleaching archaea known (Tab. 1).

Tab. 1: Overview of the most prominent bioleaching microorganisms. Listed are mesophilic & moderately thermophilic bacteria as well as thermophilic & extremely thermophilic archaea (Brandl, 2008; Donati & Sand, 2006)

Domain	Organism	Nutrition Type	Main Leaching Agent	pH Range	pH Optimum	Temperature [°C]
Mesophilic & moderately thermophilic bacteria	*Acidithiobacillus ferrooxidans*	chemolithoautotrophic	ferric iron, sulfuric acid	1.4-6.0	2.4	28-35
	Acidithiobacillus thiooxidans	chemolithoautotrophic	sulfuric acid	0.5-6.0	2.0-3.5	10-37
	Acidithiobacillus caldus	chemolithoautotrophic	sulfuric acid	1.0-3.0	2.5	42-45
	Leptospirillum ferrooxidans	chemolithoautotrophic	ferric iron	0.0-3.0	2.5-3.0	30
	Leptospirillum ferriphilum	chemolithoautotrophic	ferric iron	0.0-3.0	1.5-1.8	30-40
	Sulfobacillus thermosulfidooxidans	chemolithoautotrophic	ferric iron, sulfuric acid	1.5-5.5	2.0	20-60
Mesophilic, thermophilic & exteremly thermophilic archaea	*Ferroplasma acidiphilum*	chemolithoautotrophic	ferric iron	1.3-2.2	1.7	15-45
	Acidianus brieleyi	facultative heterotroph	sulfuric acid	1.0-4.0	1.5-3.0	45-75
	Metalloshaera sedula	chemolithoautotrophic	ferric iron, sulfuric acid	1.0-4.5	2.0-3.0	50-75
	Sulfolobus metallicus	chemolithoautotrophic	ferric iron, sulfuric acid	1.0-1.4	2.0-3.0	50-86

Currently, as a consequence of their capability to thrive at temperatures above 65°C, the potential of archaea is under intensive investigation (Norris *et al.*, 2000; Abdollahi *et al.,* 2014). Archaea of the order *Sulfolobales* are known to be involved in bioleaching (Rohwerder *et al.,* 2003), with genera like *Acidianus, Sulfolobus* and *Metallosphaera*. *Acidianus brierleyi* was the first isolated archaeon in connection with bioleaching (Brierley, 1978). It is able to grow on sulfur or ferrous iron as well as heterotrophically utilizing complex organic compounds (Segerer *et al.,* 1986). The application of *Acidianus brierleyi* in leaching processes leads to higher recovery rates than achieved with mesophilic microorganisms (Brierley, 1990). *Sulfolobus metallicus* is an obligate autotrophic archaeon oxidizing ferrous iron, reduced inorganic sulfur compounds and/or metal sufides (Huber & Stetter, 1991) This archaeon is frequently applied for the leaching of chalcopyrite (Dopson *et al.*, 2006; Jordan *et al.*, 2006). Another archaeon used in bioleaching is *Metallosphaera sedula*, an aerobic iron-and sulfur-oxidising chemolithotrophic archaeon that can also grow

on complex organics (Huber *et al.,* 1989). Mesophilic ferrous iron oxidising archaea of the order *Thermoplasmales,* genus *Ferroplasma,* have been described (Golyshina *et al.,* 2000). Although archaea have a large potential for bioleaching, their lack of a cell wall renders them sensitive towards hydrodynamic conditions, such as high shear forces (Nemati *et al.*, 2000).
There is also evidence for heterotrophic bacteria and fungi contributing to bioleaching by metal mobilisation due to enzymatic reactions, production of organic acids or by compounds with hydrophilic reactive groups. Bacteria of the genus *Bacillus* and fungi of the genus *Aspergillus* and *Penicillium* are found among these ones (Bosecker, 1997; Anjum *et al.,* 2012).

1.5 Microbial attachment and biofilm formation

Microorganisms can grow in free living “planktonic” or in “biofilm” states. Biofilms are surface-associated communities of microorganisms embedded in a matrix of extracellular polymeric substances (EPS), which mediate the adhesion and aggregation of cells, supports the cohesion of the biofilm and retention of water, and act as a protective barrier, nutrient source, energy storage and reaction space for redox reactions. EPS offers the cell a place for sorption of organic compounds and inorganic ions, enzyme activity, exchange of genetic information and export of cell compounds (Flemming and Wingender, 2010). EPS mediate the contact between the cell and the substratum and, therefore, had an important role for the leaching itself (Fig. 2). It has been shown that EPS are a prerequisite for attachment, especially the positively charged exopolymer-complexed iron(III) ions are essential for electrochemical interactions with the negatively charged pyrite surface. EPS also enlarge the reaction space for the attack of iron(III) ions (tunneling effect) on the mineral (Gehrke *et al*., 1998).
Primary attachment occurs mainly by electrostatic interactions between the positively charged cells (more precisely the EPS surrounding the cells, in which likely 2 mol negatively charged glucuronic acid residues complex 1 mol positively charged iron (III) ions resulting in a net positive charge) with the negatively charged pyrite surface (at pH 2 in sulfuric acid solution) (Gehrke *et al*., 1998). If the inoculum exceeds the available surface area, some cells may remain in the planktonic state. Interestingly this occurs even though the surface area is only less than 5 % covered by cells. Hydrophobic interactions as well as covalent bonds seem to mediate the secondary (tight) surface attachment (Vera *et al.,* 2013). The adhesion force for cells of leaching bacteria fall in the range of 0.6 up to 1.1 nN between a single cell and the surface of the mineral chalcopyrite (Schippers *et al.,* 2014).
Furthermore, attachment to areas with a low degree of crystallization is favored, proximity to such dislocations and boundaries might confer an advantage (Watling, 2006).

1.6 Chalcopyrite (bio)mining

Chalcopyrite ($CuSFe_2$) is derived from the Greek words "*chalkos*" (copper) and "*pyrites*" (strike fire) and is also known as copper pyrite (Szymanowski, 1996). It is a brassy to golden yellow colored mineral and was discovered in Polk Country in 1847 (Baba *et al.,* 2012). It is not only the most abundant copper mineral in the world (Leahy & Schwarz, 2009), but also the most stable one, because of its structural configuration ($Cu^{+}Fe^{3+}(S^{2-})_2$). This primary copper sulfide has a wide band gap (0.6 eV) between the filled valence and empty conduction bands and a high lattice energy of 17,500 kJ, both contributing to its refractory nature (Bevilaqua *et al.,* 2014).
Chalcopyrite is a known semi-conductor with a resistivity of 10^{-3} Ωm (Tshilombo *et al.,* 2002). The crystal structure consists of a simple tetragonal lattice, with each sulfur ion surrounded by four metal ions of copper and iron located on tetrahedron angles. The substitution of copper and iron by other metal atoms in the crystal lattice of natural chalcopyrite leads to the formation of n- and p-type semiconductor structures, with an energy gap of around 0.6 eV (Córdoba *et al.,* 2008a). Due to this strong lattice, it is refractory to most hydrometallurgical treatments and requires powerful oxidizing agents for dissolution (Prasad & Pandey, 1998). Equations [1] and [2] describe the dissolution of chalcopyrite, depending on whether sulfur and sulfate [1] or only sulfate is produced [2].

[1] $CuFeS_2 + {}^{5}/_{4}\, O_2 + {}^{5}/_{2}\, H_2SO_4 \rightarrow CuSO_4 + {}^{1}/_{2}\, Fe_2(SO_4)_3 + S^0 + {}^{5}/_{2}\, H_2O$

[2] $CuFeS_2 + {}^{17}/_{4}\, O_2 + {}^{1}/_{2}\, H_2SO_4 \rightarrow CuSO_4 + {}^{1}/_{2}\, Fe_2(SO_4)_3 + {}^{1}/_{2}\, H_2O$

Many oxidizing agents have been applied to dissolve chalcopyrite, such as oxygen (Padilla *et al.,* 2008), hydrogen peroxide (Mahajan *et al.,* 2007), nitric acid (Prater *et al.,* 1973), cupric copper (Hiroyoshi *et al.,* 2000), chloride (Córdoba *et al.,* 2008a), silver (Hiroyoshi *et al.,* 2002) and dichromate (Aydogan *et al.,* 2006). However, ferric iron is the most common used agent (Dutrizac, 1981; Córdoba *et al.,* 2008a).
Ferric sulfate leaching can be combined very well with bioleaching, since bacteria can regenerate the oxidant (ferric iron). Several factors including redox potential and pH influence bioleaching of chalcopyite. Mesophilic leaching (> 800 mV (SHE)) does normally not proceed as good as thermophilic leaching (680 - 700 mV (SHE)) (Petersen & Dixon, 2002; Nazari & Asselin, 2009). Considerably less copper is leached during the same time period by mesophilic than by thermophilic microorganisms (Clark *et al.,* 2006; Córdoba *et al.,* 2008c). Chalcopyrite in solution exhibiting a redox potential of less than 640 mV (SHE) is always in an active stage. In the range of 640 to 710 mV (SHE) it is a bistable system and, depending on its polarisation, active or passive. However, at redox potentials higher than 710 mV (SHE) chalcopyrite is passive and leaching is very slow (Viramontes-Gambao *et al.,* 2010). There is no general consent that microorganisms are always helpful in chalcopyrite dissolution (Third *et al.,* 2000; Stott *et al.,* 2003; Córdoba *et al.,* 2008c). During low bacterial activity (oxidation of ferrous iron) the copper oxidation is fast. Increased amounts of ferric ions do not enhance

the copper extraction rate (Third *et al.,* 2000). When the amount of ferrous iron is totally oxidized to ferric iron and the redox potential reaches 800 mV (SHE) the leaching rate turns to zero. Chalcopyrite leaching is not inhibited by the cells, but by the ferric iron and, thus, the high redox potential (Córdoba *et al.,* 2008c) which leads to jarosite deposition and passivation of the chalopyrite surface (Stott *et al.,* 2003). The addition of ferric iron depresses the leaching process, while the addition of ferrous iron enhance chalcopyrite leaching by one order of magnitude (Third *et al.,* 2000). Nevertheless, microorganisms can oxidize reduced sulfur compounds and elemental sulfur, both arising during the dissolution of chalcopyrite to sulfate, producing acid and removal of elemental sulfur from the chalcopyrite surface (Vargas et al., 2014). The pH of the leaching solution is influenced by the acid consumption of the ore, by the iron oxidation and precipitation as well as by the acid-producing oxidative reactions. Usually it is increasing during chalcopyrite dissolution (Bevilaqua *et al.,* 2014). At pH values between 2 and 3 the biologically driven iron oxidation rate is 10^4 times higher than the corresponding rate of chemical oxidation. At pH values higher than 3 iron oxidation is hindered due to precipitation and the bacterial activity is slowed down (Meruane & Vargas, 2003).

In the last 40 years intensive research has been carried out in order to identify the cause of the slow dissolution of chalcopyrite (Debernardi & Carlesi, 2013). Today there is an agreement on that extraction rate deceleration which is caused by a passivation film (Hackl *et al.,* 1995) that prevents the diffusion of the reactants to the surface (Pradhan *et al.,* 2008). However, the nature of this film is still unknown (Córdoba *et al.,* 2008a). Several hypotheses on this phenomenon have been made (Brierley & Brierley, 2013). The formation of a sulfur layer (Dutrizac, 1989), disulfides (Klauber *et al.,* 2001), polysulfides (metal-deficient sulfides) (Parker et al., 1981; Hackl et al., 1995) and the precipitation of iron hydroxy-oxides (jarosite) (Stott *et al.,* 2000; Sandström *et al.,* 2005; Córdoba *et al.,* 2008b) are the main predicted reasons for chalcopyrite passivation.

Although sulfur and disulfides are systematic products of the acidic ferric sulfate dissolution (Munoz *et al.,* 1979; Parker *et al.,* 1981; Dutrizac, 1989, Klauber *et al.,* 2001) these are constantly found on the surface of chalcopyrite. It is believed that they form a porous layer and do not hinder the dissolution of chalcopyrite (Klauber, 2008). Besides, bacteria can oxidize elemental sulfur to sulfate (Parker *et al.,* 2003).

It has been reported that the chalcopyrite dissolution proceeds through the formation of a layer of nonstoichiometric polysulfides or iron-poor sulfides ($Cu_{1-x}Fe_{1-y}S_{2-z}$ (y>x) or Cu_XS (0.7<x<1)) that react slower than a clean surface and hinder the copper extraction (Hackl et al. 1995; Klauber 2008; Nazari & Asselin, 2009). Harmer et al. (2006) explain the preferential leaching of iron over copper by the oxidation power of ferric ions in an initial stage. The initial non-stoichiometric release ratio of Fe:Cu is typically varying from ~2:1 (Linge, 1976) to ratios in the order of 4 or 5:1 (Biegler & Swift, 1979; Holliday & Richmond, 1990). On the basis of electrochemical charge balances and the nonstoichiometric preferential release of iron over copper, several authors have proposed the formation of nonstoichiometric compounds such as $Cu_{1-x}Fe_{1-y}S_{2-z}$ (y>x) and Cu_XS (0.7< x < 1) (Warren et al. 1982; Biegler and Horne 1985; Holliday and Richmond 1990; Hackl et al. 1995). The metal-deficient sulfide is a

transformed surface phase, unlike the other candidates, which can be regarded as oxidation products (Klauber, 2008).

Jarosite precipitation has been also suggested to impair the interaction between the chalcopyrite surface and the bacteria. Jarosite is readily produced (see Equation 3) in a leaching solution containing high amounts of Fe^{3+} and $SO_4{}^{2-}$ and cations like K^+, Na^+, $NH_4{}^+$ or H_3O^+ (see Equation 3), which are common nutritional species for microbial growth (Parker *et al.,* 2003).

[3] $3\ Fe_2(SO_4)_3 + (K^+; Na^+; H_3O^+)_2SO_4 + 12\ H_2O \rightarrow 2\ (K^+; Na^+; H_3O^+)Fe_3(SO_4)_2(OH)_6 \downarrow + 6\ H_2SO_4$

Along with the hydrolysis of iron, other reactions occur which can cause precipitations such as goethite (FeOOH), and, in the presence of sulfate, Schwertmannite ($Fe_8O_8(OH)_6(SO_4)$) (Bingham *et al.,* 1996). It has been reported that jarosite formation is triggered by the presence of precursors such as goethite (FeOOH) and iron hydroxides ($Fe(OH)_X$) (Klauber et al. 2001; Córdoba et al. 2008b). The formation of jarosite species strongly increases with temperature, especially above 60°C. The pH and iron concentration have an inverse influence on the formation of precipitates. At pH levels lower than 1.5 jarosite formation is largely prevented (Debernardi & Carlesi, 2013). In the presence of jarosite seeds or mineral particles aggregation nuclei can be formed which enhance jarosite precipitation (Dutrizac, 1996; Klauber, 2008). Jarosite precipitation is predominant in the pH range of 1.9–2.2 (Pradhan *et al.,* 2008). Redox potentials higher than values between 600 and 700 mV (SHE) favor Fe^{3+} precipitation as jarosite and the subsequent chalcopyrite passivation. The solubility product constant K_{sp} (10^{-11}) and the free energy of jarosite formation $\Delta G°_f$, (−3309.8 kJ/mol) indicate a very low solubility and a high stability of jarosites (Debernardi & Carlesi, 2013). Jarosite decreases the iron concentration about 35 % (Bevilaqua et al. 2014). Furthermore, a coprecipitation of copper with jarosite is also possible (Hiroyoshi et al., 1999).The decreasing of the pH or the removal of ions that favor iron precipitation could be a way to prevent ferric ion hydrolysis. However, attempts in this direction have not solved the problem of chalcopyrite passivation (Córdoba et al., 2008b). Stott et al. (2000) utilized a jarosite bioreduction step to remove this compound and recover the initial leaching rate. They partially removed the amount of jarosite (70%), but failed to increase the leaching rate, concluding that other factors than jarosite formation also affect the degradability (Debernardi & Carlesi, 2013).

Several requirements are known to enhance chalcopyrite leaching, including high temperature and low redox potential (Córdoba et al., 2008a). Chloride for example is known to enhance the leaching rate by one order of magnitude compared to ferric sulfate leaching. However, the many negative effects of chloride (i.e. affinity towards many elements, being extremely corrosive) and the higher costs of hydrochloric acid compared to hydrogen sulfide acid made it unsuitable (Córdoba et al., 2008a). Silver has been applied as catalyst for chalcopyrite dissolution. The formation of Ag_2S on the surface modifies the anodic dissolution and inhibits passivation (Watling, 2006; Córdoba et al., 2008a), but its high price and the recovery made it unsuitable for industrial applications.

Galvanic interactions between pyrite and chalcopyrite are known to enhance chalcopyrite dissolution. Pyrite provides an alternative surface for ferric iron reduction in the leach solution, which enables the system to lower the amount of ferric ions in solution and exhibit a high oxidation potential within the active anodic region of the chalcopyritic mineral. Typically, an excess of pyrite is used for effective galvanic leaching of chalcopyrite (mass ratio of 2:1 to 4:1; Nazari & Asselin, 2009).
Minerals with a more positive rest potential (more noble minerals, i.e. pyrite: 630mV) act as a galvanic cathode, while minerals with a more negative potential (chalcopyrite: 520mV, chalcocite: 440mV, covellite: 420mV) act as a functional anode (Mehta & Murr 1983; Debernardi & Carlesi, 2013).

1.7 Chalcocite and covellite (bio)mining

Chalcocite (Cu_2S) is a secondary copper (I) mineral. In 1958 Djurle discovered by X-ray analyses of the Cu-S system a second phase comprising $Cu_{1.96}S$, called djurleite. Chalcocite and djurleite are frequently mixed or intergrown with each other. The only way to distinguish between them is by X-ray diffraction analysis (Evans, 1979a). Chalcocite can also be associated with digenite ($Cu_{1.8}S$) and other intermediates ($Cu_{2-x}S$) (Miki *et al.*, 2011). There are two known structures of chalcocite: “low chalcocite” and “high chalcocite” occuring at temperatures of below or above 103.5 °C, respectively. In both structures the sulfur atoms are in hexagonal closepacking. The difference between them is the totally different arrangement of the copper atoms (Evans, 1979b). Whereas in the low chalcocite the copper atoms occupy the triangular interstices, in the high chalcocite structure the copper atoms are in disorder in the interstices (Buerger & Wuensch, 1963).
Today the most heap bioleaching operations are extracting copper from chalcocite ores (Brierley & Kuhn, 2010), ranging in extraction rates of 50-90% with mesophilic bacteria (Lee *et al.,* 2011). Due to its high copper content (67%) chalcocite is the most profitable copper ore (Davenport et al., 2002). Chalcocite leaching occurs in two distinct stages. In the first stage chalcocite is oxidized to a covellite-like form ($Cu_{1.4}S$), also called “blue-remaining covellite” [4] (Leahy *et al.,* 2007). The kinetic of the first stage is diffusion-limited (Dixon, 2000; Cooper & Dixon, 2006). The reaction has a low activation energy (25 kJ/mol) and proceeds quickly, releasing 40% of the copper (Ogbonna *et al.,* 2006). This step can be performed either by chemical leaching with acid or by bioleaching (Brierley & Kuhn, 2010).

[4] $Cu_2S + 1.6\ Fe^{3+} \rightarrow 0.8\ Cu^{2+} + 1.6\ Fe^{2+} + Cu_{1.4}S$

[5] $Cu_{1.2}S + 2.4\ Fe^{3+} \rightarrow 1.2\ Cu^{2+} + 2.4\ Fe^{2+} + S$

The second stage of chalcocite leaching [5] is the rate limiting step (Dixon, 2000; Cooper & Dixon, 2006). This step proceeds slowly and releases the remaining 60% of copper. While the temperature is not crucial in the first step, the reaction rate in the second step (requiring an activation energy of 100 kJ/mol) increases significantly with increased temperature

(Ogbonna *et al.,* 2006). The formed "blue-remaining covellite" produced in the first step cannot be solubilized by acid; therefore ferric iron as an oxidizing agent is needed (Brierley & Kuhn, 2010). Microorganisms provide the ferric iron for the dissolution of the "blue-remaining covellite" (Keeling *et al.,* 2006), which is very slow (Brierley & Brierley, 2013). They also maintain a high oxidation-reduction potential (Brierley & Kuhn, 2010). Some authors have observed a third step in chalcocite leaching provoking an intermediate product, i.e. copper-rich covellite ($Cu_{1.2}S$), before the conversion to "blue-remaining covellite" (Grizo *et al.* 1982). Miki *et al.* 2011 showed that the initial dissolution of chalcocite at a redox potential of 500 mV does not proceed beyond a copper release of 50 %, because covellite cannot be leached at this redox potential.
Several factors have to be considered for a good recovery of copper from chalcocite ores. Solubilized fluoride from minerals like creedite ($Ca_3Al_2SO_4(F,OH)_{10}{\cdot}2(H_2O)$), and gearksutite ($CaAl(OH)F_4{\cdot}H_2O$) as well as chloride (Zammit *et al.* 2011) can inhibit the growth of microorganisms (Brierley & Kuhn, 2010). Also the particle size is important. The smaller the grains are, the easier the ferric iron can diffuse into the ore (Ogabonna *et al.,* 2006). The presence of pyrite (FeS_2) can promote the leaching of chalcocite by providing ferric iron (Cooper & Dixon, 2006). In general, the focus of research has shifted from mesophilic microorganisms to thermophilic ones in order to benefit from their rapid leaching kinetics at increased temperatures (Hawkes *et al.,* 2006). Also the aeration of leaching operations shows positive effects on bioleaching (Leahy *et al.,* 2007).
In general, chalcocite is a well known copper ore used since many years for heap leaching operations all over the world, which operate with native microorganisms in all temperature ranges.

Covellite (CuS), also called as cupric sulfide, is a deep blue mineral with a copper content of 66.4%. The name derives from the discoverer Nicola Covelli (1790-1829). It occurs as flexible plates in the form of coatings or disseminations within other copper minerals (Wilson, 1982). Covellite is, besides its interest for the copper industry, also used for pigments, as a catalyst and as a solar radiation absorber (Lee *et al.,* 2008).
As already mentioned, covellite and the secondary covellite "blue-remaining covellite" are intermediate products of the leaching of chalcocite (Miki *et al.,* 2011). The difference between covellite and "blue-remaining covellite" is their copper content. "Blue-remaining covellite" contains 1.5-2.0 % more copper then covellite. The two minerals can be distinguished visually by their color in immersion oil (Putnis *et al.,* 1977). Normal covellite shows a red-orange anisotropic color, whereas "blue-remaining covellite" shows a blue color. The high activation energies for "blue-remaining covellite" (51.8±6.2 kJ) and covellite (58.3±13.7 kJ) suggest that the rate-limiting step is rather a surface reaction than a diffusion process in the aqueous leaching solution (Walsh & Rimstidt, 1986). Besides "blue-remaining covellite" there are also other types of secondary covellite known like geerite ($Cu_{1.6}S$) and yarrowite ($Cu_{1.1}S$) (Whiteside & Globe, 1986). Secondary covellites are more reactive than covellite itself (Acar et al., 2005) and can be chemically leached with ferric iron (at an effective concentration of 0.25 M to 1 M; Miki *et al.,* 2011).

Studies showed that leaching of covellite at elevated temperature increases the extraction rate. After 346 days of bioleaching copper extraction rates of 20 % using mesophiles and 80 % with thermophiles were achieved (Acar *et al*., 2005). Umrania & Joshi (2002) demonstrated that the copper extraction rate can be increased using thermophilic bacteria adapted to high copper concentrations. Lee *et al.* (2011) reported that covellite and enargite cannot be efficiently leached by mesophilic bacteria. However, thermophilic bacteria at 65 °C can extract 60-98 % of the copper. Sakaguchi *et al.* (1976) described the optimum conditions for leaching of synthetic covellite with *At. ferrooxidans* requiring a pH of 2.3 and a ferric iron concentration of 0.004 to 0.2 M at a temperature of 35 °C. The leaching of covellite is possible in the absence of a chemical lixiviant, when the bacteria are in direct contact with the ore (Pogliani *et al.,* 1990). Nevertheless, the addition of pyrite in a ratio of 1:1 or 1:2 (covellite:pyrite, w/w) can enhance the leaching of covellite (Zhou *et al.,* 2007). The application of *At. thiooxidans* and in mixed species cultures with *At. ferrooxidans* showed that this combination is a good option for covellite leaching. *At. ferrooxidans* maintains the ferric iron level high and *At. thiooxidans* maintains the pH low. Also the application of *At. thiooxidans* with supplements of ferrous iron has been proven to be useful for covellite leaching (Curutchet *et al.,* 1995; Curutchet *et al.,* 1996; Donati *et al.,* 1996). First, ferrous iron is oxidized by dissolved oxygen to ferric iron. Then, the elemental sulfur covering the surface is removed by the metabolic action of the bacteria allowing the ferric iron to get in contact with the oxidable mineral.

In general there are few publications available about leaching of covellite, since it is considered to be an intermediate product of chalcocite leaching. Extensive research on covellite leaching remains to be done. Covellite is very rarely found as pure mineral. It is mostly found in complex ores inter-grown with other copper minerals. It is also formed during chalcopyrite and chalcocite leaching as intermediate product.

1.8 Cuprous copper

The presence of cuprous ions in leaching environments has previously been reported by Muir *et al.,* 1975 (cupric chloride leaching) and Mc Donald *et al,.* 1984 (electrorefining involving organic nitriles) and recently by Anwar *et al.,* 2000 (sulfate-based leaching). Cuprous ions are known to be not stable in aquatic environments (Matoha *et al.,* 2005), unless there are complexed by chloride (Parker *et al.,* 1981) or by solvents like CH_3CN (Georgopoulos *et al.,* 2001) or acetonitrile (Altermatt *et al.,* 1968). In seawater, the presence of cuprous ions has been also reported (Moffettt *et al.,* 1988; Millereo *et al.,* 1991). The formation of cuprous copper is assumed due to the reduction of cupric copper by sunlight-generated free radicals like superoxide or hydrogen peroxide (Millero *et al.,* 1991, Kiuane & Shinghasmanon *et al,.* 2011). The same mechanisms has been held responsible for cuprous copper found in urban river water, involving Fe(II)/Fe(III) and dissolved and colloidal organic compounds and low oxygen conditions (Glazewski *et al.,* 1996). In fog water 0.1-1 pM cuprous copper was found (4 - 90% of total copper), and in this case its occurrence is accounted to the reduction of cupric copper by sulfite (Xue *et al.,* 1991). The same

phenomena has been observed in rain water (Kieber *et al.,* 2004). The uptake of copper by rainbow trout gills had been reported due to a reduction of cupric to cuprous copper prior to membrane transport (Bopp *et al.,* 2008), it was found that this reaction was catalyzed by sulfhydryl groups (Felsenfeld, 1960; Bogdanova *et al.,* 1991). The reason for the existence of cuprous copper in sulfate-based solution could be related to iron species (Matocha *et al.,* 2005). A reduction of cupric ions to cuprous ions is possible by ferrous iron (Wegwe, 1999).
Cuprous copper is known to be more toxic than cupric copper (Beswick *et al.,* 1976; Holland & White, 1988). Its toxcicity towards *Escherichia coli* (Beswick *et al.,* 1976; Singh *et al.,* 2004), spermatozoa (Holland & White, 1988) and mammalian cells (Hesselwood *et al.,* 1978) has been tested. While cuprous copper showed a toxic effect towards spermatozoa within the range of 0.2-0.4 mg/mL, cuprous copper showed toxic effect within a range of 0.08-0.16 mg/L (Holland & White, 1988). It is not clear yet, if the higher toxcicity derives from the cuprous copper itself or due to the radical generation due to its oxidation (Hesselwood *et al.,* 1789; Holland & White, 1988).

1.9 Calorimetry

All chemical, physical and biological processes are either producing or consuming heat. Therefore calorimetry is a good technique for the study of thermal activities in terms of heat, heat flow and heat capacity (Russel *et al.*, 2009).
In 1780, Lavoisier and de Laplace performed the first calorimetric experiment. They placed a guinea pig into an adiabatic lined container with ice on the inside. From the amount of melted water and the body weight of the guinea pig a power of 3 $^{W}/_{kg\ of\ body\ weight}$ could be calculated (von Stockar & Marison, 1989). Human beings possess a typical energy output of 1.3 $^{W}/_{kg\ of\ body\ weight}$, but microorganisms can produce much more energy. A resting yeast culture is able to produce 25 $^{W}/_{kg\ of\ dry\ mass}$ and when starting growth even more than 1 $^{kW}/_{kg\ of\ dry\ mass}$ (von Stockar & Birou, 1989). In 1856, the first microbiological calorimetric experiment was realised by Dubrunfaut. He measured the heat output of sugar fermentation by using 21.400 L of culture fluid and 3.5t of sugar (von Stockar & Marison, 1989).
Since the late seventies of the 20th century, microcalorimetry is experiencing stronger demand due to the strongly improved sensitivity of the instrumentation (Maskow *et al.,* 2010). The general application fields of this technique are listed in Tab. 2.

Tab. 2: Main application fields of calorimetry (von Stockar & Marison, 1989).

1.	**Process development work**
	Experimental determination of process cooling requirements
	Monitoring biological activity as a function of process conditions, detecting unsuspected special events & anomalies such as limitations, product formation, diauxic growth, etc.
	Medium optimization: Identification of limiting factors & inhibitors
	Rapid and quantitative characterization of growth kinetics
	Investigation of limiting mass transfer phenomena
2.	**Bioprocess & bioreactor control**
	Use of heat dissipation measurements at production scale as an on-line "probe" for indirect determination of • Biomass concentration & growth • Product formation • State of culture
	Use of on-line heat dissipation measurement at production scale together with other on-line signals for computer-aided of bioreactors
3.	**Research**
	Enzyme-level kinetics of culture
	Induction-repression kinetics in continuous culture
	Growth energetic & biothermodynamics

As mentioned in Table 2, microcalorimetry plays a significant role in environmental sciences, investigations on trace elements, living organisms, solute-solvent interactions, sorption processes and the identification of the stability of technical products (Sand, 1978; Russel *et al*., 2009). Additionally, it is a powerful tool for the monitoring of microbial activities (Tab. 3). As few as 10^3 to 10^4 living bacterial cells are sufficient to obtain a real-time signal, which can be related to the cell number and their metabolic activity (Braissant *et al.,* 2010).

Tab. 3: Application fields of isothermal microcalorimetry for the monitoring of microbial activities (Braissant *et al.,* 2010).

Heat flow measurements with isothermal microcalorimetry to monitor microbial activity
Detection of microorganisms
Discrimination of microorganisms
Evaluation of microbial processes
Determination of the performance of antimicrobial agents

Recent trends in calorimetric developments are megacalorimetry for larger amounts of sample quantities, chip-calorimetry for very low amounts of sample, high-throughput calorimetry for enthalpy arrays and finally ultra-sensitive calorimetry and photocalorimetry for the investigation of photosynthesis (Maskow *et al.,* 2010).

1.9.1 Fundamentals of calorimetry

Besides the broad scale of different calorimeter types, all are based on two measurement principles. In adiabatic calorimeters heat quantity is measured through the temperature change and the heat capacity of the calorimetric vessel and its content. Therefore no heat exchange between the calorimetric vessel and the surrounding should happen (Wadsö, 1997). The second measuring principle is the heat conduction or compensation calorimetry. It is based on a controlled flow of heat from the calorimeter vessel to the surrounding (heat

sink), usually represented by an aluminium block. A thermopile positioned between sample container and the heat sink is used as a sensor for the heat flow (Wadsö, 1997). The temperature difference between vessel and heat sink correlates with an electrical potential U which may directly deduct from the heat flow sensor. The thermal power is calculated by the Tian equation [6].

[6] $P = \varepsilon (U + \tau \frac{dU}{dt})$

[7] $P = \varepsilon U$

[8] $P = \varepsilon \int U dt$

ε : Calibration constant

τ : Time constant

U : Thermal power

$\frac{dU}{dt}$: Time derivative of the thermopile potential

Equation [7] is characterising the steady-state process. The heat quantity (P) is given by the integral of the potential (U) [8] (Wadsö, 1997).

1.9.2 Isothermal microcalorimetry

Heat conduction microcalorimeters are usually equipped with a semi-conducting thermopile, often called Peltier element. These calorimeters are termed isothermal calorimeters, even though they are not truly isothermal, as they allow small temperature variations of up to 0.1°C (Braissant *et al.,* 2010). To increase sensitivity and accuracy, isothermal microcalorimeters are operated in the so-called `twin instrument' mode, where the reaction vessel and an inert reference are measured at the same time (von Stockar & Marison, 1989). The main advantage of microcalorimetry is the relatively low number of active cells (10^3 to 10^5 cells/ml) needed for detection. This is substantially lower than the detection limit of spectrophotometers. Within a performance range of 15 to 300°C, the thermal accuracy is typically 0.02°C (Braissant *et al.,* 2010). Isothermal microcalorimetry can provide continuous real-time data; this method is also called isothermal titration calorimetry and is applied for molecular and kinetic studies (Wadsö, 1997). Microcalorimetric measurements are non-destructive, non-invasive and do not need special sample preparation (Buchholz *et al.,* 2010). Nevertheless, there are some drawbacks. Microcalorimetry needs an equilibration time of at least one hour, also oxygen limitation and metabolic waste accumulation in the reaction vessels may occur. Another issue is the nonspecific signal, which is the sum of all reactions occurring in the measuring cylinder (Braissant *et al.,* 2010). The advantages and disadvantages of isothermal microcalorimetry are summarized in Tab. 4.

Tab. 4: Advantages and disadvantages of isothermal microcalorimetry (Braissant *et al.*, 2010).

Advantages	Disadvantages
Low detection limit	Equilibrium time
High accuracy	O_2 limiting conditions in reaction vessel
Continuous real-time detection	Accumulation of metabolic waste
Non-destructive	
Non-invasive	
No sample preparation necessary	

1.9.3 Microcalorimetry and biomining

In the past mainly organic consuming microorganisms were chosen as model organisms for microcalorimetric investigations (Forrest, 1970), basically because heterotrophic organisms generate more heat than autotrophic ones. As a consequence, only few studies are published in this context dealing with bioleaching organisms known to grow mostly chemolithoautotrophically. One of the first studies using chemolithoautotrophic bacteria was performed by Dessers *et al.* in 1970 determining the free energy efficiency of *Nitrobacter winogradskyi*.

The first calorimetric study of a bioleaching bacterium was done in 1969 by Lees *et al*., investigating the free-energy changes during iron oxidation by acidithiobacilli. Ten years later Goodman & Ralph (1979) did the first experiments applied to the field of mineral degradation. They studied the metabolic activity of acidithiobacilli with the intention to develop a library of thermogram patterns of acidithiobacilli. Thus, natural isolates could be identified more rapidly. In 1987 Schröter and Sand introduced microcalorimetry as a technique for the fast evaluation of microbial activity associated with mineral degradation. They showed that thiosulfate oxidation proceeds via three steps and also could produce strain-specific thermograms. Two years later they published strain-specific thermograms for leptospirilli and nitrifying bacteria (Schröter and Sand, 1989).

Although the potential of microcalorimetry for the investigation of biofilm activity has been known since the 1980s, the first approach using calorimetry involving microbial attachment was performed by Weppen *et al.* in 1991, who measured the oxygen demand of surface-grown cells. In 1994 Wentzien *et al.* investigated biofilm formation of *Thiomonas intermedius* and *Paracoccus versutus* with microcalorimetry. Recently, Buchholz *et al.* (2010) reported on metabolic changes of attached and planktonic cells by calorimetric measurements.

In 1991 Hallmann investigated the influence of acidophilic chemoorganotrophic microorganisms on bioleaching by microcalorimetry. The author measured the activity of microorganisms in mineral ore samples and used in parallel the “most probable number“ technique (MPN) for the quantification of viable cells. It was concluded that the heat output of an ore sample is proportional to the cell number. Mixed consortia exhibit a heat output in the range of 20-100 $^{\mu W}/_{h}$. In 1993 Schröter and Sand demonstrated the application of microcalorimetry on the estimation of the degradability of ores and bacterial leaching activity. In the same year Schröter also performed microcalorimetric measurements on iron

(II) and thiosulfate oxidizing chemolithotrophic bacteria. The author detected that heat flow can be directly correlated to the iron oxidation rate (Schröter, 1993). One year later Wentzien *et al.* (1994) investigated the degradation of thiosulfate and tetrathionate by acidithiobacilli species with the application of a flow through-microcalorimeter. In 1995 Schippers *et al.* used microcalorimetry to assess the microbial bioleaching activity in a uranium mine waste heap. Three years later Schippers (1998) analysed the sulfur chemistry of bioleaching of metal sulfides with the help of microcalorimety. Rohwerder (1998) applied microcalorimetry to investigate the thermodynamics of pyrite leaching. He determined the reaction energies of pyrite dissolution by acidophilic bacteria, measuring reaction energies for pyrite dissolution ranging from -1100 to -1600 $^{kJ}/_{mol}$, which is very close to the calculated value of -1546 $^{kJ}/_{mol}$. In pure *At. ferrooxidans* cultures and mixed cultures the theoretical and measured values showed no significant differences. In contrast, *L. ferrooxidans* cultures showed reaction energies up to 200 $^{kJ}/_{mol}$ lower than the theoretical ones. This effect can be correlated with the inability of *L. ferrooxidans* to oxidize reduced sulfur compounds (Rohwerder *et al.*, 1998). Elberling *et al.* in 2000 investigated the bacterial and chemical oxidation of pyrite at low temperatures by measuring the microbial activity with microcalorimetry. In several other studies (Schippers *et al.,* 2000; Schippers *et al.,* 2001; Kock & Schippers, 2006; Sand *et al.* 2007; Schippers *et al.,* 2007) microcalorimetry was used to assess mine tailings and acid mine drainage with respect to their microbial bioleaching activity.

Summarizing, microcalorimetry represents a suitable and promising method for the detection and quantification of bioleaching, since bacteria are producing heat during bioleaching and the detection limit is very low (Buchholz *et al.,* 2010). Since chemolithoautotrophs are difficult to grow on solid agar plates the MPN technique must be done using serial dilutions of liquid medium including several weeks of cultivation (Brierley, 1978). A direct counting of the bacteria can be difficult, because some of them are frequently forming biofilms onto the mineral substrate (Vera *et al*., 2013). Application of microcalorimetry for the evaluation of microbial activity in mineral ore samples is not as time consuming as the MPN technique. In addition, it is more accurate, since the MPN has a range of variation of about 10% in natural samples due to the selective growth of microorganisms (Hallmann, 1991). Microbial activity can directly be correlated with iron and sulfur oxidation, based on the fact that the standard reaction enthalpy for the dissolution of mineral sulfides can be measured, i.e. the standard reaction enthalpy of pyrite oxidation has been determined to -1546 $^{kJ}/_{mol}$ (Schippers, 1998). In 1995 Rohwerder correlated the heat output to the bacterial leaching rate with defined cultures. He calculated 100µW of heat production equals to a pyrite oxidation rate of 6.0 $^{\mu mol}/_{day}$ for *At. ferrooxidans* and a mixed culture of *At. thiooxidans* and *L. ferrooxidans*. For 100 µW of heat production a leaching rate of 7.5 $^{\mu mol}/_{day}$ was calculated for *L. ferrooxidans* (Rohwerder, 1995). Even though microcalorimetry is not as widespread in use as other techniques, it certainly possesses the potential for a rapid determination of microbial activity in bioleaching. Furthermore, the heat production can be correlated with the iron and sulfur oxidation rates.

2. Objectives of this work

The aim of this work is the establishment of a microcalorimetric determination method to assign the chemical and biological degradation of copper sulfides such as chalcopyrite, chalcocite and covellite by means of measuring the thermal energy released in the process of the respective material conversion. This includes:

i) the screening of microorganisms for an efficient copper sulfide bioleaching

ii) the verification of microcalorimetric determinations with different ore types and bioleaching cultures growing at various temperatures and the thermodynamic calculation of the reaction energy $\Delta_r U$ by means of the measured heat output and the determined iron, copper and sulfate ions in the leachate

iii) High resolution microscopy observation of the biofilm formation by bioleaching cultures on chalcopyrite

iv) observations on the cuprous copper stability within the leachates of different cultures and abiotic leaching experiments at different temperatures.

3. Materials and methods

3.1 Strains and growth conditions

Selected strains and enrichment cultures were obtained from the culture collection of the department of Aquatic Biotechnology, Biofilm Centre, University of Duisburg-Essen. Microorganisms used in this study are listed in Tab. 5.

Tab. 5: Microorganisms used in this study.

Strain	Origin	Reference
Acidithiobacillus ferrooxidans ATCC 53993^T	copper deposit, Armenia	Premuzic & Lin (1994)
Acidithiobacillus caldus DSM 8584^T	tetrathionate enrichment culture, Warwick, United Kingdom	Hallberg & Lindström (1995)
Leptospirillum ferriphilum DSM 14647^T	enrichment culture, Peru, 1984	Sand *et al*. (1992), Coram & Rawlings (2000)
Sulfobacillus thermosulfidooxidans DSM 9293^T	spontaneously heated ore deposit, Eastern Kazakhstan	Golovacheva & Karavaiko (1978)
Sulfolobus metallicus DSM 6482^T	solfataric field, Iceland	Huber & Stetter (1991)

Enrichment cultures used in this study are shown in Tab. 6.

Tab. 6: Enrichment cultures used in this study.

Culture	Origin	Reference
M6	volcanic sediments Copahue, Argentina	Provided by Dr. M. Vera
RAM	ore deposit, Rammelsberg, Germany	Provided by Prof. Dr. A. Schippers
AS	copper mine, Kerman, Iran	Ahmadi *et al*. (2011)

All strains and microorganisms were cultivated in autotrophic basal salt solution (pH 1.5) according to Mackintosh (1978) supplemented with 4 g/L ferrous iron or 1 g/L sulfur flower. Ferrous iron solution was prepared separately from the media with ferrous(II)sulfate. Media and solutions were sterilized at 121°, 1.2 bar for 90 minutes. For media prepared with sulfur supplementation the initial pH was adjusted to 2.5 by addition of potassium hydroxide prior to autoclaving. Medium containing sulfur was prepared and sterilized at a temperature of 110°C.

All cultures were shaken at 150 rpm in darkness, except of *Sulfolobus metallicus* cultures, which were shaken at 100 rpm. The culture conditions of the microorganisms used are shown in Tab. 7.

Tab. 7: Culture conditions of the microorganisms used in this study.

Strain/ culture	Growth substrate	Supplementation	Growth temperature
At. ferrooxidans	iron/ sulfur	-	28 °C
At. caldus	sulfur	-	45° C
L. ferriphilum	iron	-	37 °C
Sb. thermosulfidooxidans	iron/ sulfur	0.02% yeast	45 °C
S. metallicus	iron/sulfur	0.02% yeast	65 °C
M6	iron	-	28 °C
RAM	iron	-	28 °C
AS	sulfur	-	45 °C

Stock cultures were grown in 100 mL narrow-neck Erlenmeyer flasks containing 50 mL basal salt solution. All cultures were grown until the substrate was almost consumed. The oxidation of ferrous to ferric iron was indicated by a change in color from clear green to rusty red. Sulfur oxidation was indicated by a drop of the pH and the suspension of the sulfur. Pre- and mass cultures were incubated with an inoculum of 10 % (v/v) in 1L (500 mL medium) or 5 L conical shoulder bottles (4.5 L medium). To ensure convenient oxygen supply mass cultures were aerated and stirred.

Batch cultures were prepared with 1 % chalcopyrite in 5 L conical shoulder bottles (4.5 L medium), which were aerated and stirred. Cells were harvested for experiments and bottles were refilled with basal salt solution (pH 1.5). Excessive cells were returned back to the batch culture.

3.2 Cell harvest and cell count determination

Cultures were harvested and washed with basal salt solution by centrifugation (Thermos Scientific, Sorvall RC 6+) at 7500 rpm at 20°C. Cell pellets were resuspended in basal salt solution. Short spins were given in order to eliminate iron or sulfur residuals.

The planktonic cell counts were determined by a Thoma hemocytometer (depth: 0.1 mm, area of smallest squares: 0.0025 mm^2) and a light microscopy (Leica DMLS, Wetzlar GmbH) in phase contrast mode with 400x magnification.

3.3 Sulfide minerals

The minerals used in this study were all in the particle size of max. 200 µm. Pyrite was boiled with 6 N HCl for 30 minutes, rinsed with deionized water to pH neutrality and washed twice with acetone (purity degree p.a.) to remove oxidation products (Moses *et al*., 1987). After washing it was dried at 60 °C. Aliquots were dry sterilized under nitrogen atmosphere at 120 °C for 48 hours and stored.

Chalcopyrite, chalcocite and covellite were sterilized under nitrogen atmosphere by heat sterilization (100 °C) and stored.

In this study the mineral sulfides chalcopyrite, chalcocite, covellite and pyrite were used. The contents of copper and iron in the minerals are shown in Tab. 8. A full element analysis of the mineral sulfides was performed by X-ray spectroscopy (XRS; Tab. 20 appendix). This method is not suitable for the assessment of sulfur because it may be easily volatised and elements such as copper, iron and zinc may be lost in the platinum crucible (Norrish & Thompson, 1990).

Tab. 8: Copper and iron percentage in the mineral sulfides used in this study. Analysed with ICP-MS (aqua regia digestion) by the BGR, Hannover.

Mineral	Origin	Copper [%]	Iron [%]
Chalcopyrite	Siegerland, Germany	35.10	29.87
Chalcopyrite	Harz, Germany	29.54	29.43
Chalcopyrite	Sweden	32.38	30.75
Chalcopyrite	Peru	36.52	32.95
Chalcocite		46.80	14.08
Covellite		50.90	3.36
Pyrite containing copper sulfide	Romania	9.59	33.40
Pyrite	BGRM, France	0.77	48.52

3.4 Analytical methods

3.4.1 pH measurement

The pH values were determined with a digital pH meter (Model pH 537, WTW, inLab® 422 Combination Semi-micro pH Electrode, Mettler Toledo).

3.4.2 Redox potential measurement

The oxidation/reduction potential (ORP) was measured with a digital redox meter (Model pH 537, WTW, inLab® RedoxMicro silver chloride Electrode). For the conversion to the potential of a standard hydrogen electrode (SHE) 207 mV have to be added to the values measured by a Ag/AgCl electrode.

3.4.3 Ferrous and total iron determination

Iron ions were quantified according to the German standard methods of examination of water, waste water and sludge; cations; determination of iron DIN 38406-1 (Anonymous, 1984a). Ferrous ions form a red complex with phenathrolinium and were photometrically measured at a wavelength of 492 nm. Total iron was determined (in a second test series) after addition of hydroxylamine (reducing ferric to ferrous iron). Ferrous and total iron concentrations have been measured in triplicates within microtiter plates (BRANDplates® pureGrade™) with a UV-Vis spectrophotometer (TECAN, Infinite pro® 200).

3.4.4 Cuprous and total copper determination

Copper ions were determined by flame atom absorption spectrometry (AAS) at a wavelength of 324,7 nm (Perkin Elmer 1100B) according to the German standard methods of

examination of water, waste water and sludge; cations; determination of iron DIN 38406-7 (Anonymous, 1984b).
In order to distinguish between cuprous and cupric ions a photometric determination according to the protocol of Anwar *et al.* (2000) based on cuproine (Hoste, 1950) was done. Cuproine forms with monovalent copper a purple complex, which can be measured at a wavelength of 560 nm. Total copper was determined by prior reduction of cupric ions with hydroxylamine to monovalent copper. Interferences with other ions, especially trivalent ions have been avoided by complexation with tartric acid (Hoste et al., 1953). Measurements were performed in triplicates within microtiter plates (BRANDplates® pureGrade™) with a UV-Vis spectrophotometer (TECAN, Infinite pro® 200).

3.4.5 Sulfate determination

The concentration of sulfate was determined by ion exchange chromatography (IC). A Dionex DX-500 system in combination with an eluent generator (EG50), a conductivity detector (CD20) and an autosampler (AS3500) were used. The system was controlled using the chromatographic software Chromeleon Version 6.7. An analytical column with anion exchange resin as stationary phase (IonPac AS17, 2 x 250 mm, Dionex USA) and a guard column (IonPac AG17, 50 mm, Dionex, USA) were used. A flow rate of 0.25 mL/min and a suppressor current of 50 mA were applied for the measurement. The injection volume of the autosampler was 10 µL. Potassium hydroxide was used as eluent. The gradient system was as followed: 0-2.5 min 10 mM KOH; 2.5-3.5 min 20 mM KOH; 3.5-4.5 min 30 mM KOH; 4.5-5.5 min 40 mM KOH; 5.5-6.5 min 50 mM KOH, 6.5-8.5 min 10 mM KOH).
Samples were diluted 1:10 with 5 mM phosphate buffer (40% 50 mM KH_2PO_4, 60% 50 mM K_2HPO_4, pH 7) to avoid (metal) precipitation in the column and incubated for 30 min. Afterwards samples were centrifuged (Biofuge, Heraeus Sepatech) for 10 minutes at 8000 rpm. The supernatants were further diluted 1:10 with bidistilled water and then measured. Samples were measured in duplicates.

3.4.6 Sulfur determination

Sulfur was extracted by ethanol and measured according to Schippers *et al*. (1996) by high-performance liquid chromatography (HPLC). The HPLC (Kontron 400 series) was equipped with a type 465 autosampler, type 425 gradient generator, type 422 pump, type 440 diode array detector and a separation column (EC 125/4 NUCLEODUR 100-5 C18, Macherey-Nagel, Germany). Methanol (p.a.) was used as mobile phase with a flow rate of 1.2 mL/ min. Data were processed by integrated software DS 450-MT1-EMS V.1.32.

3.5 Visualisation of biofilm formation

Attachment and biofilm formation on sulfide minerals were visualized by Confocal-Laser-Scanning-Microscopy (CLSM). A laser scanning module (LSM 510 Carl Zeiss®Jena) coupled to an inverted Axiovert 100 MBP microscope (Zeiss®) was used. The microscope was operated

with LSM 510 Release 3.2 software (Zeiss®). Image manipulation was performed using the PC software ImageJ (http://rsbweb.nih.gov./ij/).
Cells were stained with the nucleic acid stain Syto®9 (Thermo Fisher Scientific, Life Technologies) according to Bellenberg *et al*., 2012.

3.6 Microcalorimetry

For the determination of the reaction energies by microcalorimetry (TAM III; TA Instruments) mineral batch cultures were filtered through 0.45 µm filter capable of retaining the microorganisms. Aliquotes of 0.3 to 1 g of the filter cake (including the bacteria) for calorimetric measurements were used directly after filtering.
Samples were measured in duplicate at the respective temperature for a time period of 4-12 hours. After a release of 4-6 J per sample, while the heat output decreased by around 20% as compared to the initial value, the measurement was terminated. Samples were measured in 3 mL ampoules. After measurements the dry weights were determined. All results are given in $^{\mu W}/_{g}$ dry mineral.
To make statements about the matching of the thermal energy measured by microcalorimetry and the predicted reaction energy by thermodynamics, the following calculations have been done (Tab. 9). For the calculations either a period of constant heat output has been chosen or the heat output of a reasonable time period has been averaged.

Tab. 9: Exemplary calculation of the reaction energy $\Delta_r U$ of chalcopyrite dissolution on the basis of microcaloric data and the concentration of released copper ions in the leachate.

<table>
<tr><td>1) A period of constant heat output has to be chosen</td><td>e.g. 250 $^{\mu W}/_{g}$ during day 0 to 4</td><td rowspan="2">3) The amount of total copper ions (released and dissolved by leaching during the period chosen for gathering the heat output) has to be converted into a oxidation rate $^{mmol}/_{g*d}$ (leaching period 4 d, leachate volume 30 mL, amount of mineral 3 g)</td><td rowspan="2">e.g. 16 mM, means 0.04 $^{mM}/_{g*d}$</td></tr>
<tr><td>2) The heat output (calculated per gram mineral) has to be converted into thermal energy Joule (J = W x sec) per day by multiplication with 10^{-6} (W) and 86400 (sec)</td><td>21.6 $^{J}/_{g*d}$</td></tr>
<tr><td colspan="4">4) To calculate the reaction energy released by the dissolution of 1 mole chalcopyrite (according to stoichiometry equivalent to one mole released copper ions), the thermal energy of the heat output ($^{J}/_{g*d}$) has to be divided by the copper oxidation rate ($^{mM}/_{g*d}$)</td></tr>
<tr><td colspan="4">calculated reaction energy $\Delta_r U$ (exothermic reaction) = -540 kJ/mol</td></tr>
</table>

Comparing the calculated reaction energy (based on empirical data as mentioned above) to the standard reaction energy $\Delta_r H^f$ of chalcopyrite dissolution given in the literature (ratio of calculated reaction energy to standard reaction energy) a statement about the copper recovery rate in the leachate can be made. Neglecting pressure and volume (during mineral

dissolution only solutions and solids are involved) $\Delta_r H^f$ can be equate with the reaction energy $\Delta_r U$. Ratios calculated to values less than 1 characterize the mineral being not fully degradable, whereas values exceeding 1 refer to precipitations of the degradation product (turning inaccessible for analytical assessment and causing the measuring parameter to become underestimated).
For chalcopyrite dissolution two equations can be applied, depending on whether the oxidation of chalcopyrite is producing elemental sulfur [9] and/or sulfate [10].

[9] $CuFeS_2 + {}^5/_4\ O_2 + {}^5/_2\ H_2SO_4 \rightarrow CuSO_4 + ½\ Fe_2(SO_4)_3 + 2\ S^0 + {}^5/_2\ H_2O$ $\quad \mathbf{\Delta_r H^f = -508\ {}^{kJ}/_{mol}}$

[10] $CuFeS_2 + {}^{17}/_4\ O_2 + ½\ H_2SO_4 \rightarrow CuSO_4 + ½\ Fe_2(SO_4)_3 + ½\ H_2O$ $\quad \mathbf{\Delta_r H^f = -1755\ {}^{kJ}/_{mol}}$

Pyrite is dissolved to iron and sulfate [11].

[11] $FeS_2 + 3.75\ O_2 + 0.5\ H_2SO_4 \rightarrow 2\ SO_4^{2-} + Fe^{3+} + H^+$ $\quad \mathbf{\Delta_r H^f = -1546\ {}^{kJ}/_{mol}}$

Chalcocite dissolution proceeds through two steps. Approximately 40 % copper is dissolved in the first step [12] and the remaining 60 % are dissolved by the second step [13]. While the first step is very fast, the second one is considerably slower (Ogbonna *et al*., 2006)

[12] $Cu_2S + H_2SO_4 + 0.5\ Fe^{3+} \rightarrow CuS + CuSO_4 + H_2O$ $\quad \mathbf{\Delta_r H^f = -211.66\ {}^{kJ}/_{mol}}$

[13] $CuS + 0.5\ Fe_2(SO_4)_3 \rightarrow CuSO_4 + FeSO_4 + 0.5\ S$ $\quad \mathbf{\Delta_r H^f = -362.32\ {}^{kJ}/_{mol}}$

4. RESULTS

4.1 Screening of appropriate microorganisms

Chalcopyrite bioleaching is known to be time consuming and also associated with low copper recoveries. Most obstacles originate from the refractory nature of the ore. Before microcalorimetric experiments were conducted, a proper selection of microorganisms was made. For this purpose four microorganisms with different growth temperature optima were chosen. *Acidithiobacillus ferrooxidans*, one of the most studied bioleaching microorganisms, is able to oxidize iron and sulfur and grows at mesophilic conditions (28 °C). In this study the type strain ATCC 53993 was used because of its high resistance towards copper (up to 100 mM, Orellana e*t al.* 2011). In the moderate thermophilic range (45°C) two bacterial species were selected, the iron oxidizer *Leptospirillum ferriphilum* (DSM 14647) and the Gram positive bacterium *Sulfobacillus thermosulfidooxidans* (DSM 9293) able to oxidize both iron and sulfur compounds while growing chemomixotrophically with yeast extract addition. To cover also the thermophilic temperature range (65 °C), the iron and sulfur oxidizing archaeon *Sulfolobus metallicus* (DSM 6482) was selected.

Leaching of a pyritic ore. To show that the selected microorganisms are capable of leaching a copper containing mineral, experiments with a pyrite ore enriched with copper sulfides were conducted (Fig. 4).

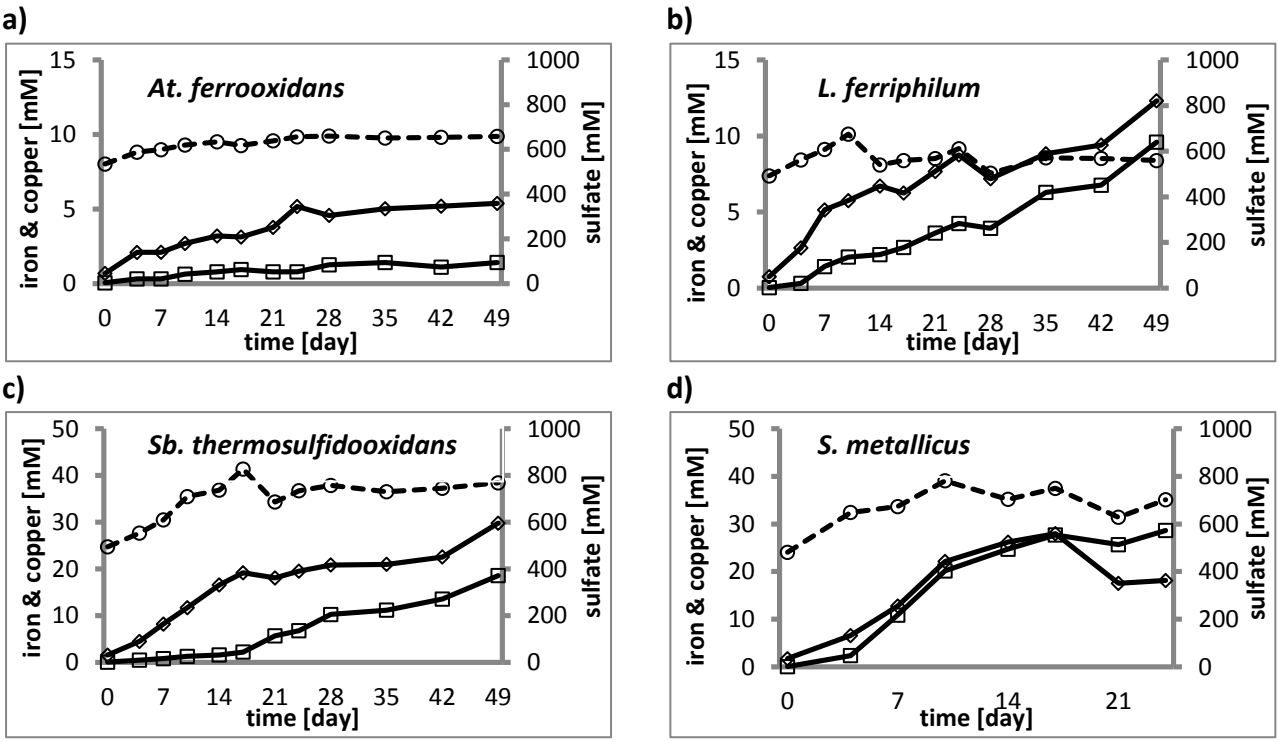

Fig. 4: Solubilized copper (□), iron (◊) and sulfate ions (○) during bioleaching of pyrite containing copper sulfides by a) *At. ferrooxidans* (28°C), b) *L. ferriphilum* (45°C), c) *Sb. thermosulfidooxidans* (45°C) or d) *S. metallicus* (65°C). Leaching assays with 50 ml basal salt solution (pH 1.5) and a mineral load of 2 % pyrite containing copper sulfides (Romania ore, 62-200 µm) shaken at 120 rpm; in total 30 mM Cu and 119 mM Fe could have been recovered.

In the temperature range of 28 to 65°C copper recoveries of 5 % to 96 % could be obtained. *At. ferrooxidans* leached 5 % copper, besides 5 % iron. In the moderate thermophilic range, *L. ferriphilum* leached 32 % copper and 11 % iron, whereas *Sb. thermosulfidooxidans* recovered 62 % copper and 25 % iron. *S. metallicus* extracted 96 % copper and 15 % iron within 25 days. As shown in Fig. 34 (given in the appendix), the counts of planktonic cells were generally decreasing during the experiment. The pH nearly remained stable in all assays (approximately 1.5) except for *S. metallicus* (increasing to values of max. 2.3) The ORP of all assays decreased after 21 days to values between 400 mV and 450 mV (Fig. 34, appendix).

Leaching of chalcopyrite. In Fig. 5 the leaching efficiencies of *At. ferrooxidans*, *L. ferriphilum*, *Sb. thermosulfidooxidans* and *S. metallicus* on pure chalcopyrite are shown.
In all four assays the pH increased with time (Fig. 35a, appendix). In *Sb. thermosulfidooxidans* and *At. ferrooxidans* assays the pH increased within two weeks to values between 3 and 4. Within the *L. ferriphilum* assays a pH of 3 could be measured after 70 days. For *S. metallicus* the pH increased to 2.5 during the first 4 weeks, then decreased slightly to a value of 2.
The cell counts of *At. ferrooxidans* and *L. ferriphilum* decreased constantly during the experiment from 10^8 to 10^6 cells/ mL. *Sb. thermosulfidooxidans* and *S. metallicus* showed a slight increase of cell counts (Fig. 35b, appendix).

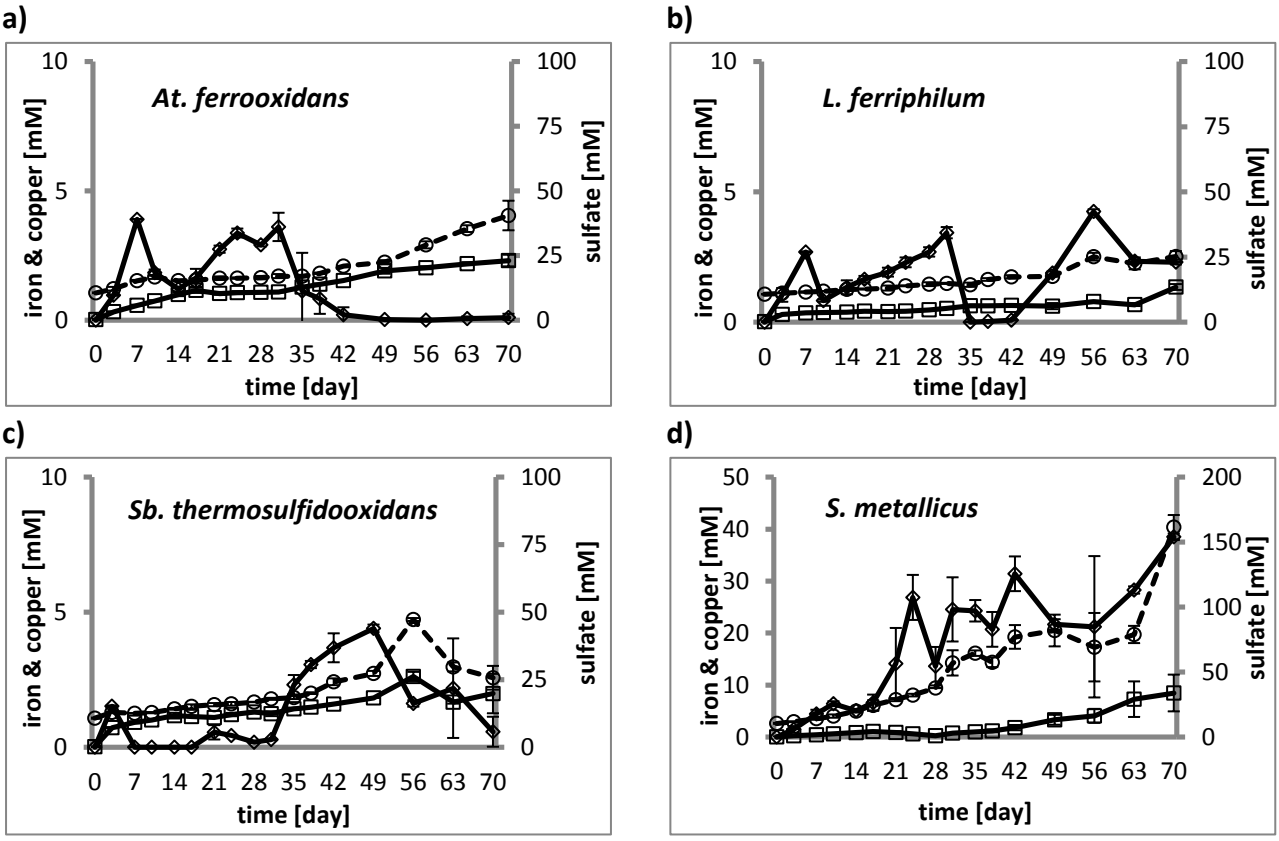

Fig. 5: Solubilized copper (□), iron (◊) and sulfate ions (○) during bioleaching of chalcopyrite containing by a) *At. ferrooxidans* (28°C), b) *L. ferriphilum* (45°C), c) *Sb. thermosulfidooxidans* (45°C) or d) *S. metallicus* (65°C). Leaching assays with 30 mL basal salt solution (pH 1.8) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) shaken at 120 rpm; in total 553 mM Cu and 520 mM Fe could have been recovered.

The ORP of *At. ferrooxidans*, *L. ferriphilum* and *Sb. thermosulfidooxidans* assays decreased from 600 mV at the beginning to values in a range of 300 mV to 400 mV. In cultures of *S. metallicus* the ORP decreased from 450 mV to 350 mV during the experiment (Fig. 35c, appendix).
With all microorganisms used in this study low recoveries of 0.24 % to 1.5 % copper were achieved (Fig. 5). Iron was not leached constantly. In *At. ferrooxidans* assays after 6 weeks iron was no longer detectable (Fig. 5a), while within the *Sb. thermosulfidooxidans* and *L. ferriphilum* assays during the first 5 weeks only negligible or little amounts of iron in solution could be measured (Fig. 5b and c). Relatively high amounts of iron could be detected in the leachate *of S. metallicus* (Fig. 5d).

Improvement of chalcopyrite leaching. Several improvement strategies for the enhancement of chalcopyrite leaching were tested (Fig. 36 and 37, appendix). The classic leaching setup with an initial pH of the basal salt solution of 1.8 was compared to a setup with an initial pH of 1.5 and to a setup with additional phosphate supplementation (1 mM phosphate) at an initial pH of 1.8. Tab. 10 shows the total recovery of copper and iron after seven weeks of leaching with *At. ferrooxidans*, *L. ferriphilum*, *Sb. thermosulfidooxidans* or *S. metallicus*.

Tab. 10: Copper and iron recoveries after 7 weeks of chalcopyrite bioleaching by *At. ferrooxidans* (28°C), *L. ferriphilum* (45°C), *Sb. thermosulfidooxidans* (45°C) or *S. metallicus* (65°C). Leaching assays with 100 mL (pH 1.8 with and without additional phosphate) or 500 mL (pH 1.5) basal salt solution; with a mineral load of 2 % (Harz ore, 62-200 µm) (pH 1.8 or pH 1.5)) or 10 % chalcopyrite (pH 1.8 with additional 1 mM phosphate) shaken at 120 rpm each. (n.a. = not available).

	Initial pH 1.8		Initial pH 1.8 (additional phosphate)		Initial pH 1.5	
	Fe recovery [%]	**Cu recovery [%]**	**Fe recovery [%]**	**Cu recovery [%]**	**Fe recovery [%]**	**Cu recovery [%]**
At. ferrooxidans	2.4	2.7	8.7	7.4	1.1	1.0
L. ferriphilum	4.3	4.7	3.1	7.3	n.a.	n.a.
Sb. thermosulfidooxidans	5.0	11.0	2.2	6.8	2.2	1.0
S. metallicus	1.2	11.0	2.4	10.7	18.2	12.6

Phosphate supplementation showed an enhancement in copper and iron recovery for chalcopyrite leaching with *At. ferrooxidans* and *L. ferriphilum*. For *Sb. thermosulfidooxidans* an initial pH of 1.8 seemed to be the best. An initial pH of 1.5 had a positive effect on chalcopyrite leaching with *S. metallicus*.
However, the selected microorganisms still showed low metal recoveries. Instead of using pure cultures, experiments with mesophilic and moderate thermophilic enrichment cultures were conducted (Fig. 38, 39 and 40, appendix). In order to adapt the microorganisms to chalcopyrite, continuously growing cultures on chalcopyrite were prepared.
Tab. 11 shows the total recovery of copper after 7 weeks of leaching with *At. ferrooxidans*, *Sb. thermosulfidooxidans* and the enrichments M6, RAM and AS, compared with adapted *At. ferroxidans* and the enrichment AS.

Tab. 11: Copper recoveries after 7 weeks of chalcopyrite bioleaching by *At.* ferrooxidans (28°C), or the enrichments M6 (28°C), RAM (28°C) or AS (45°C), as well as with adapted *At. ferroxidans* and the adapted enrichment AS. Leaching assays with 30 ml basal salt solution and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) shaken at 120 rpm. Sterile controls leached 1.8 % (28°C) or 3.0 % (45°C) copper.

Culture	Cu recovery [%]
At. ferrooxidans	4.5
Adaped *At. ferrooxidans*	5.0
Mesophilic enrichment M6	2.7
Mesophilic enrichment RAM	3.6
Moderate thermophilic enrichment culture AS	5.5
Adapted moderate thermophilic enrichment culture AS	7.3

While enrichment cultures did not show regularly higher copper recoveries, cultures adapted to chalcopyrite generally showed enhanced copper extraction. Further experiments to improve the leaching efficiency of the selected microorganisms were not conducted. For the calorimetric experiments basal salt solution with a pH of 1.5 was used, because pH values higher than 1.8 favor the precipitation of iron hydroxides. Cultures were adapted to the pH of 1.5 and showed higher copper recoveries as compared to cultures which have not been adapted before (Tab. 11).

4.2 Degradability of chalcopyrite ores

During this study, different chalcopyrite ores have been used. A test on the degradability of the ores had been conducted (Fig. 41 and 42, appendix). In Tab. 12 the copper and iron recoveries are shown.

Tab. 12: Copper and iron recoveries after 3 weeks of chemical leaching at 45°C and bioleaching of chalcopyrite ores originating from different sampling sites by the adapted moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt solution plus 10% mineral load (particle size 62-200 µm) incubated at 45 °C and shaken at 120 rpm.

Origin of ore	Copper recovery [%]		Iron recovery [%]	
	AS	Sterile control	AS	Sterile control
Siegerland, Germany	11.0	3.0	3.9	3.5
Peru	3.6	2.3	3.4	2.4
Sweden	4.0	1.3	3.8	3.0
Harz, Germany	3.9	2.0	3.4	3.1
Romania (Pyritic)	27.0	3.6	5.6	2.5

Variations in copper recovery were observed. The ore from Siegerland, Germany, showed the highest copper recovery (11 %) among the pure chalcopyrite ores. From the pyritic Romanian ore 27 % copper could be recovered. However, iron recoveries from all ores were similar. Generally no sulfur was detected during the leaching experiment (leading to equation 2 of chalcopyrite dissolution for calculation of the reaction energy), except for the thermophilic assays inoculated

with *S. metallicus* (leading to equation 1 of chalcopyrite dissolution for calculation of the reaction energy).

In Fig. 6 the evolution of heat output of the five different ores is displayed. While the heat output of the Siegerland ore increased during the first two weeks and then decreased drastically, the heat output of the Romanian pyritic ore increased from 150 µW/g to 500 µW/g after three weeks. During the leaching of the Siegerland ore 25 mM copper were dissolved after 14 days and after 21 days 55 mM copper could be measured in the leachate. The other ores emitted approximately 200 µW/g during the time period between day 3 and day 21. Chemical controls showed a heat output of below 10 µW (Fig. 43, appendix).

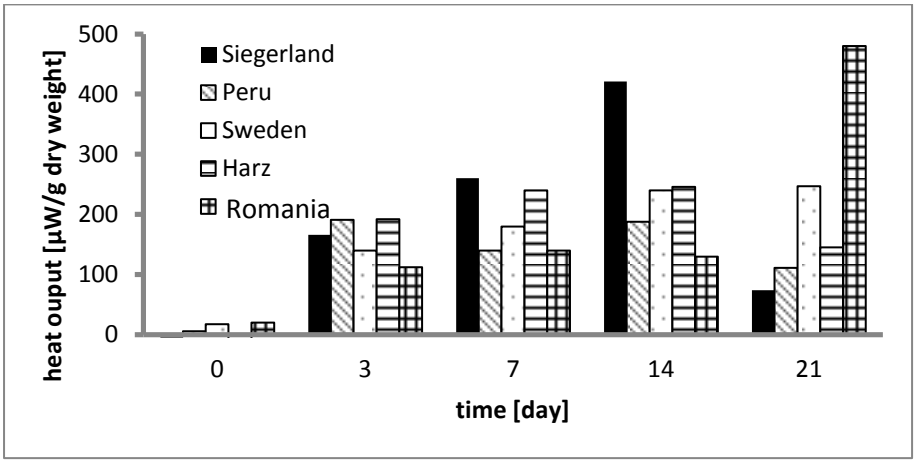

Fig. 6: Microcalorimetric determination of the microbial activity during bioleaching of chalcopyrite ores originated from different sampling sites by the moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (particle size 62-200 µm) incubated at 45 °C and shaken at 120 rpm.

The amounts of degradation products like copper, iron and sulfate ions calculated on the basis of weight loss measurements on the chalcopyrite material were compared to the concentrations of these ions measured in the leachate (Tab. 13). In some experiments the dissolution of the mineral did not reveal stoichiometrical quantities of the leaching products. In some assays red precipitates could be seen.

In case of leaching chalcopyrite with a mineral load of 10 % over a period of two weeks by moderate thermophilic or thermophilic microorganisms the calculated amount of released copper correlated with the measured concentration in the leachate. This was also true for iron and sulfur calculations after moderate thermophilic leaching. However, in case of thermophilic leaching 50% Fe and 25% S could be rediscovered. During mesophilic leaching only 20 % of the calculated amount of copper and iron was detected and only 50 % of the calculated sulfur concentration was measured.

When chalcopyrite was leached with a mineral load of 1 %, the iron concentration was lower than the estimated iron concentration at all temperature ranges measured. Although the measured copper concentration was considerably lower than the calculated concentration for the mesophilic and moderate thermophilc temperature ranges. For all temperatures measured the iron concentration was lower than expected. The concentration of total sulfur matches the calculated vlues but only for the moderate thermophilic and thermophilic leaching experiments. It was not the

case for the mesophilic leaching experiments. Although the gravimetric measurements were conducted very carefully, an overestimation of the theoretically determined copper, iron and sulfur amounts could happen due to scaling error or precipitations.

For the following experiments the amount of released copper ions was taken as basis for thermodynamic calculations on the mineral dissolution rate. Results in Tab. 13 clearly show that copper recovery proceeds better then iron recovery.

Tab. 13: Amount of released copper, iron and sulfur as calculated on the basis of the weight loss of chalcopyrite compared to the concentrations of these degradation products measured in the leachate after 14 days leaching. Leaching assays with 3g chalcopyrite (Siegerland ore, 62-200 µm) and 30 mL basal salt solution (pH 1.5; 10 % mineral load) or 1g chalcopyrite (Siegerland ore, 62-200 µm) and 50 mL basal salt solution (pH 1.5; 1 % mineral load) each shaken at 120 rpm and inoculated with *At. ferrooxidans* (28 °C), the moderate thermophilic enrichment culture AS (45 °C) or *S. metallicus* (65 °C).

Leaching microorganism	Loss (g)	Copper		Iron		Total sulfur	
		calculated [mM]	measured [mM]	calculated [mM]	measured [mM]	calculated [mM]	measured [mM]
10 % mineral load							
***At. ferrooxidans* (28°C)**	0.1674	30.84	6.54	29.76	6.55	61.08	30.21
Moderate thermophilic enrichment culture AS (45°C)	0.1555	28.65	30.45	27.65	32.14	56.74	15.41
***S. metallicus* (65°C)**	0.1206	22.22	23.10	21.44	9.52	44.01	28.39
1 % mineral load							
***At. ferrooxidans* (28°C)**	0.1266	14.00	1.78	13.51	2.32	27.72	45.91
Moderate thermophilic enrichment culture AS (45°C)	0.1224	24.76	5.98	23.90	3.57	49.04	42.76
***S. metallicus* (65°C)**	0.1526	16.87	19.95	16.28	11.10	33.41	30.36

In Tab. 14 the oxidation rates for copper and the respective thermal outputs are shown.

Tab. 14: Copper oxidation rate and release of thermal energy during chalcopyrite leaching with the moderate thermophilic enrichment culture AS.

Origin of ore	Copper		Heat output [µW/d]	Thermal energy of heat output [J/ g*d]	calculated reaction energy Δ,U [kJ/mol]	Ratio of calculated to standard reaction energy
	Recovery [mM]	Oxidation rate [mmol/ g*d]				
Siegerland, Germany	26.19	0.026	350	30.24	996	**0.57**
Peru	12.54	0.013	200	17.28	1920	**1.09**
Sweden	19.52	0.019	200	17.28	1818	**1.04**
Harz, Germany	10.4	0.011	200	17.28	2160	**1.23**
Romania (pyritic)	12.07	0.012	200	17.28	1440	**0.82**

Between 57 % and 100 % of the standard reaction energy trapped in the chalcopyrite mineral could be gained by microcalorimetry.

The experiment showed that microcalorimetry is principally suitable for the detection and quantification of chalcopyrite degradation by microorganisms. In case of the ores originated from Peru or Sweden data fluctuations resulted in slight overestimation of the respective reaction energy. The reaction energy of the Harz ore appeared to be significantly inflated and refers to (copper containing) precipitations in the leachate. The relatively low reaction energy of the ores from Siegerland and Romania indicated a higher refractory nature of these minerals.

4.3 Calorimetric measurements of chalcopyrite degradation at 28 °C

Figure 7 shows the values for dissolved copper, iron, and sulfate as well as the heat output during chemical leaching or bioleaching with *At. ferrooxidans*, the mesophilic enrichment M6, or the mesophilic enrichment RAM at 28 °C.
The cell counts (starting at 10^8 cells/mL) in all assays decreased during the first days, but then increased up to above 10^8 cells/mL. The pH increased to values of 1.6 to 1.7 after one week and remained stable for the following two weeks (Fig. 44, appendix).

Cells of *At. ferrooxidans* leached 15 mM copper, the M6 enrichment 12 mM and the RAM enrichment 18 mM during three weeks (equivalent to a total copper recoveries of 2.7, 2.2 and 3.3 %, respectively). In the sterile control assay 5 mM copper was measured. This means a total copper recovery of below 1%. However, considerably less iron ions compared to copper ions were dissolved in all assays (Fig. 7 and Fig. 45, appendix).

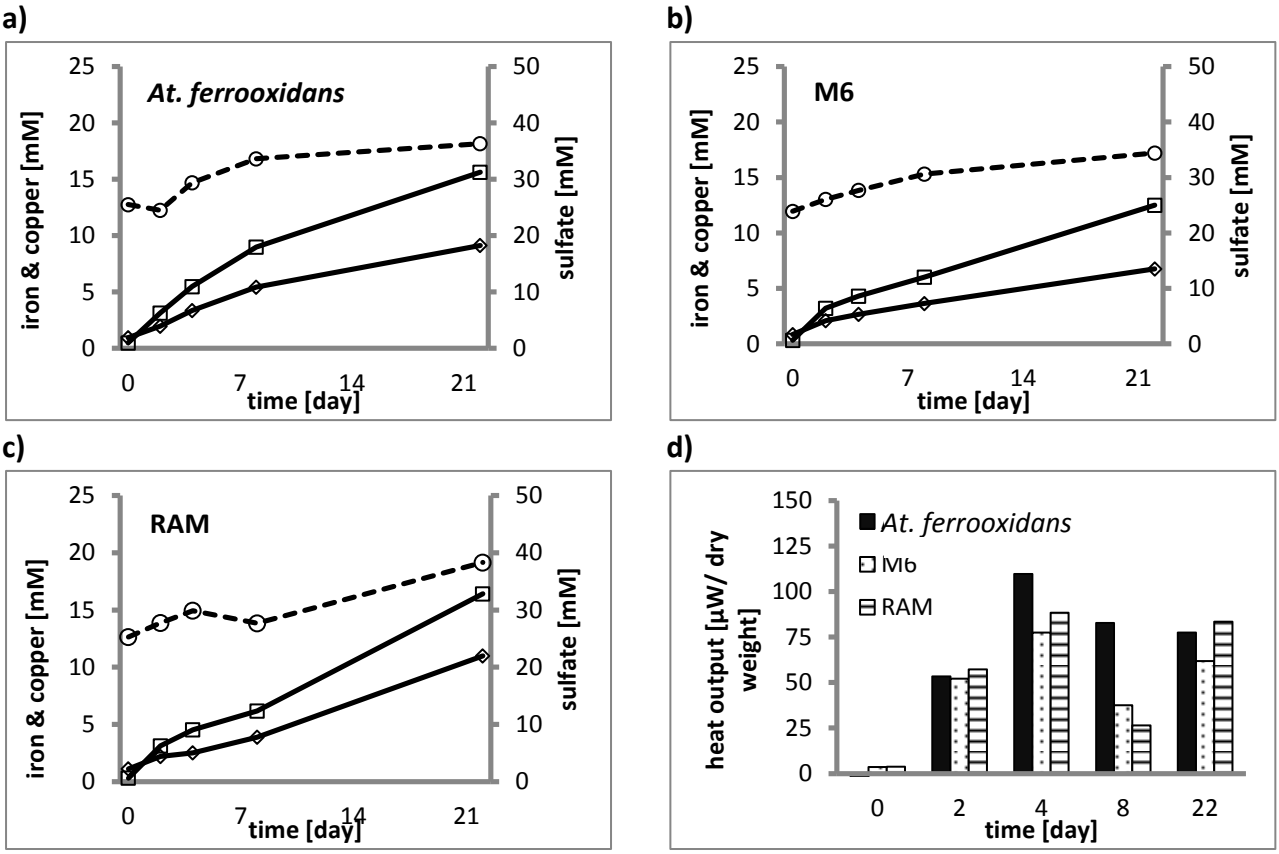

Fig. 7: Bioleaching of chalcopyrite by a) *At. ferrooxidans* or the mesophilic enrichment cultures b) M6 or c) RAM. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 28°C and shaken at 100 rpm; iron (◊), copper (□) and sulfate (○); **d)** microcalorimetric determination of the microbial activity during the leaching of chalcopyrite with *At. ferrooxidans*, the enrichment M6, or the enrichment RAM at 28°C; in total 553 mM Cu and 520 mM Fe could have been recovered; in total 553 mM Cu and 520 mM Fe could have been recovered.

In Tab. 15 the copper oxidation rate and the thermal output during bioleaching of chalcopyrite under mesophilic conditions are shown.

Tab. 15: Copper oxidation rate and release of thermal energy during chalcopyrite leaching with *At. ferrooxidans* or the mesophilic enrichment cultures M6 or RAM at 28 °C.

Cultures	Heat output [µW/d]	Thermal energy of heat output [J/g*d]	Copper oxidation rate [mmol/g*d]	Ratio of calculated to standard reaction energy
At. ferrooxidans	90	7.77	0.006	**0.78**
M6	60	5.18	0.004	**0.72**
RAM	70	6.05	0.006	**0.56**

Considering the amount of released copper ions 56 % to 78 % of the standard reaction energy trapped in the mineral could be detected by microcalorimetry.

For the comparison of chalcopyrite and pyrite, bioleaching experiments were conducted with both minerals and the mesophilic enrichment culture RAM (appendix, Fig. 46 and 47). In the chalcopyrite assays the pH increased during the first week to 3 and the cell counts decreased after one week

considerably. During leaching the pH in the pyrite assays decreased slightly and the cell counts increased from 10^8 cells/ mL to $5*10^8$ cells/ mL within 3 weeks (Fig. 45, appendix).
The enrichment culture RAM could dissolve 40 mM copper and 10 mM iron during chalcopyrite bioleaching, this is a total recovery of 7.3 % copper and 1.9 % iron after 2 weeks of leaching. With the help of microcalorimetry 24 % of the standard reaction energy could be gathered regarding the copper oxidation rate for the first 9 days. For pyrite leaching 100 % of the standard reaction energy could be detected regarding the amount of released iron ions during the first 5 days (Fig. 47, appendix).

In order to check if the pre-culture conditions have an effect on the mineral dissolution, tests were conducted with *At. ferrooxidans* pre-grown on chalcopyrite or sulfur (Fig. 8).

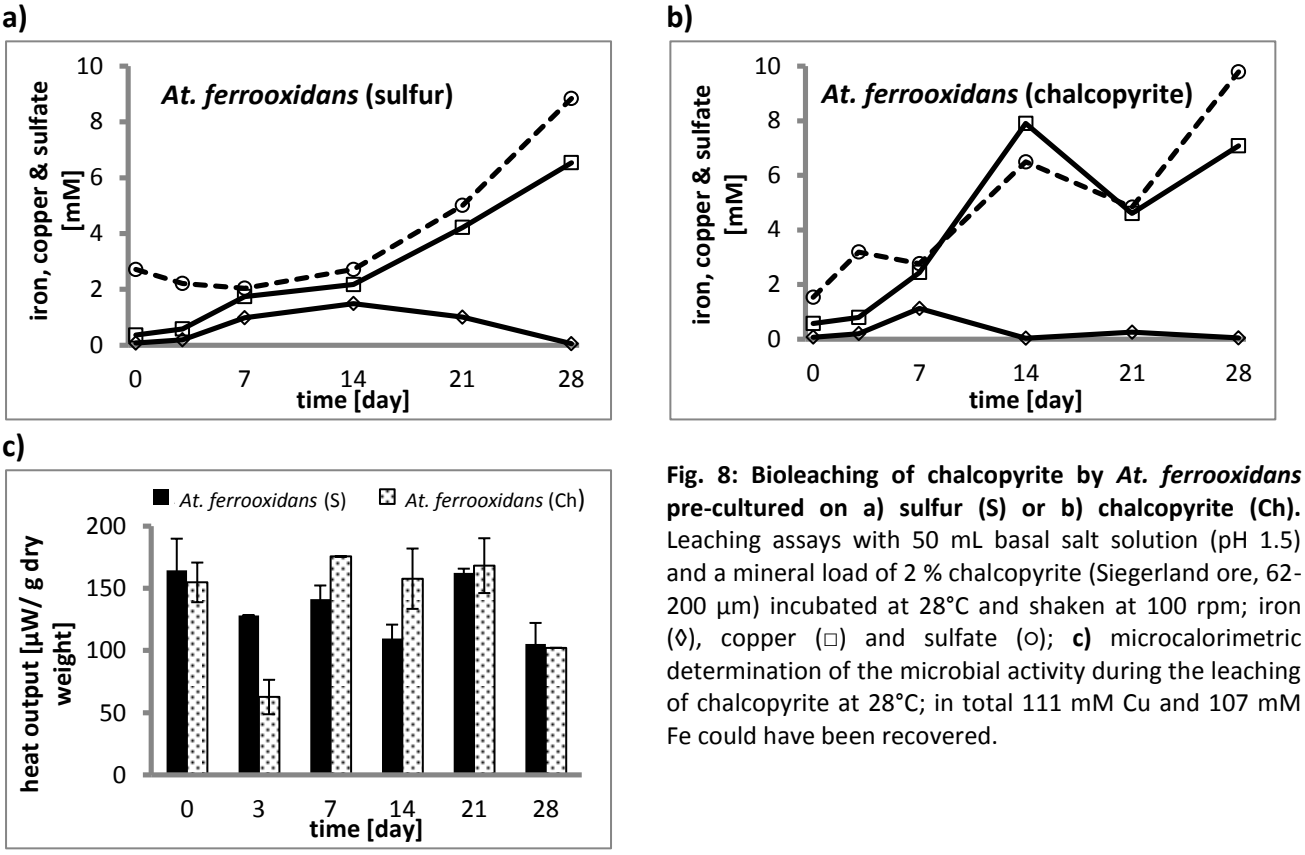

Fig. 8: Bioleaching of chalcopyrite by *At. ferrooxidans* pre-cultured on a) sulfur (S) or b) chalcopyrite (Ch). Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 2 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 28°C and shaken at 100 rpm; iron (◊), copper (□) and sulfate (○); **c)** microcalorimetric determination of the microbial activity during the leaching of chalcopyrite at 28°C; in total 111 mM Cu and 107 mM Fe could have been recovered.

The cell counts of the differently pre-grown *At. ferrooxidans* cultures decreased during the first week and increased afterwards. The pH of all assays remained almost constant (Fig. 48, appendix). In total 5.4 % copper were leached by the culture pre-grown on sulfur, 7.2 % by the culture pre-grown on chalcopyrite and 2.7 % by the sterile control at 28 °C (Fig. 49, appendix). The content of solubilized iron decreased to zero in the sulfur pre-grown culture after four weeks and in the chalcopyrite pre-grown culture after two weeks. In the sterile control assay 1.4 % iron was detectable.
Considering the copper oxidation rate and the heat output during 14 days of leaching 82 % of the standard reaction energy were gathered microcalorimetrically for the sulfur pre-grown cells. For chalcopyrite pre-grown cells 74 % was detected (Tab. 16).

Tab. 16: Copper oxidation rate and release of thermal energy during chalcopyrite leaching with *At. ferrooxidans* pre-cultivated on sulfur or chalcopyrite.

Cultures	Heat output [µW/d]	Thermal energy of heat output [J/ g*d]	Copper oxidation rate [mmol/ g*d]	Ratio calculated to standard reaction energy
***At. ferrooxidans* (sulfur)**	150	12.96	0.009	**0.82**
***At. ferrooxidans* (chalcopyrite)**	150	12.96	0.01	**0.74**

4.4 Calorimetric measurements of chalcopyrite degradation at 45°C

The cell counts in the moderate thermophilic enrichment culture AS assays increased during the experiment from 10^8 cells/ mL to above $5*10^8$ cells/ mL for assays inoculated with cells pre-cultured on sulfur or chalcopyrite. In the pyrite pre-grown assays the cell counts were decreasing during the first two weeks to almost 10^7 cells/ mL, but then increased to 10^8 cells/ mL. The pH increased only marginally (Fig. 50 and 51, appendix).

By leaching of chalcopyrite with sulfur, chalcopyrite or pyrite pre-grown cells at 45 °C in all assays 6 % of the total copper was recovered. However, this also occurred in the sterile control (Fig. 9).

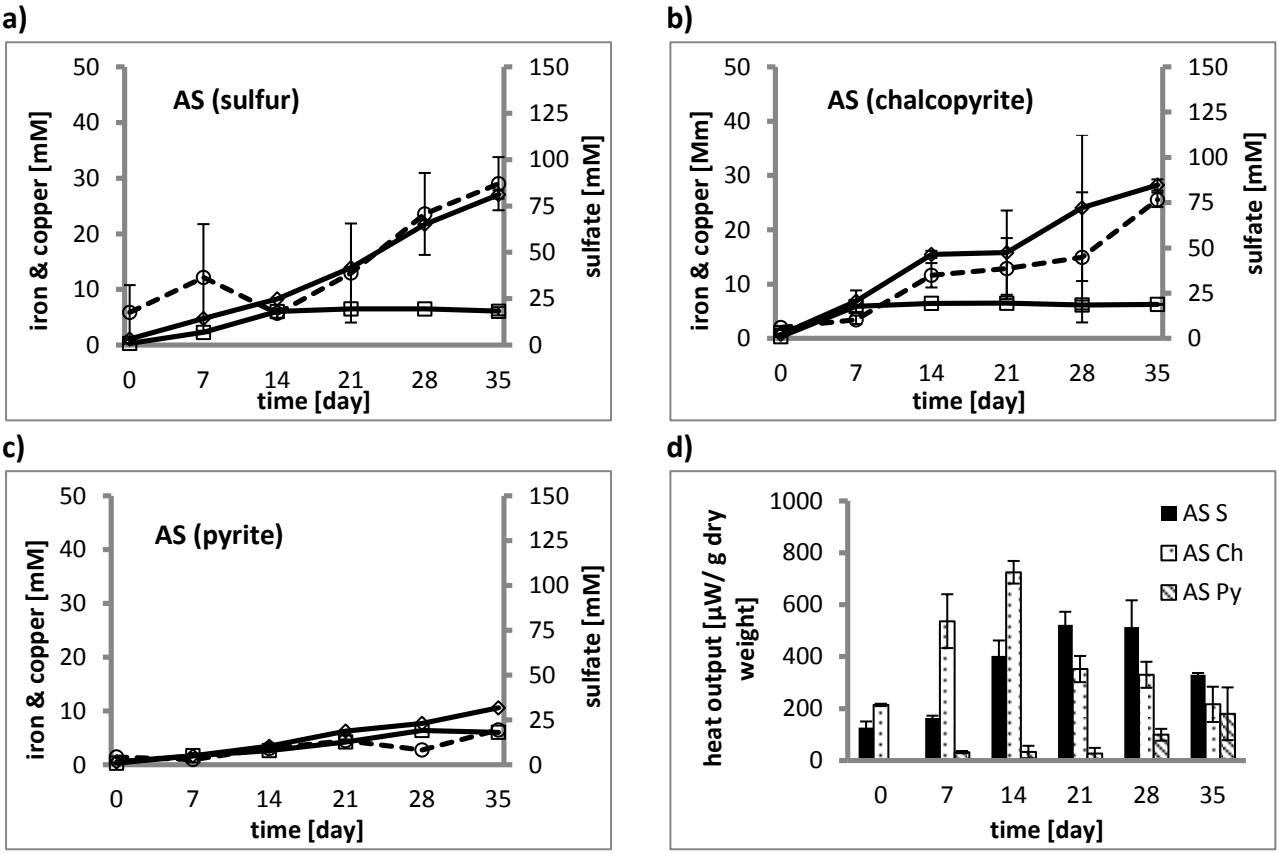

Fig. 9: Bioleaching of chalcopyrite by the moderate thermophilic enrichment culture AS pre-cultured on a) sulfur (S), b) chalcopyrite (Ch) or c) pyrite (Py). Leaching assays with 50 mL basal salt solution and a mineral load of 2 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 45°C and shaken at 100 rpm; solubilized ions in the leachate, iron (◊), copper (□) and sulfate (○); **d)** microcalorimetric determination of the microbial activity during the leaching of chalcopyrite at 45°; in total 111 mM Cu and 107 mM Fe could have been recovered.

Calculating the thermal output during the first 3 weeks (on the basis of the copper oxidation rate), 65 %, 106 % and 62 % of the standard reaction energy could be measured for the sulfur, chalcopyrite or the pyrite pre-grown cultures, respectively (Tab. 17).

Tab. 17: Copper oxidation rates and release of thermal energy during chalcopyrite leaching with the moderate thermophilic enrichment culture AS pre-cultivated on sulfur, chalcopyrite or pyrite.

Cultures	Heat output [µW/d]	Thermal energy of heat output [J/ g*d]	Copper oxidation rate [mmol/ g*d]	Ratio calculated to standard reaction energy
AS (sulfur)	300	25.92	0.009	**0.65**
AS (chalcopyrite)	450	38.88	0.009	**1.06**
AS (pyrite)	50	4.32	0.004	**0.62**

The influence of the pre-growth conditions on pyrite degradation was verified with the moderate thermophilic enrichment culture AS (Fig. 52 and 53, appendix). Cell counts of the sulfur and chalcopyrite assays with pre-grown cells remained stable during the experiment, while the cell counts of the pyrite assays with pre-grown cultures decreased dramatically to 10^6 cells/ mL after one week. The pH decreased within time (Fig. 52, appendix).
In assays with sulfur, chalcopyrite or pyrite pre-grown cells after 3 weeks of leaching at 45°C a total iron recovery of 23 %, 10 % or 3 %, respectively, was measured (Fig. 53, appendix). The sterile control showed a recovery of 3 %.

The results of the experiments with adapted or non-adapted cultures to chalcopyrite of the moderate thermophilic enrichment culture AS are shown in Fig. 10.

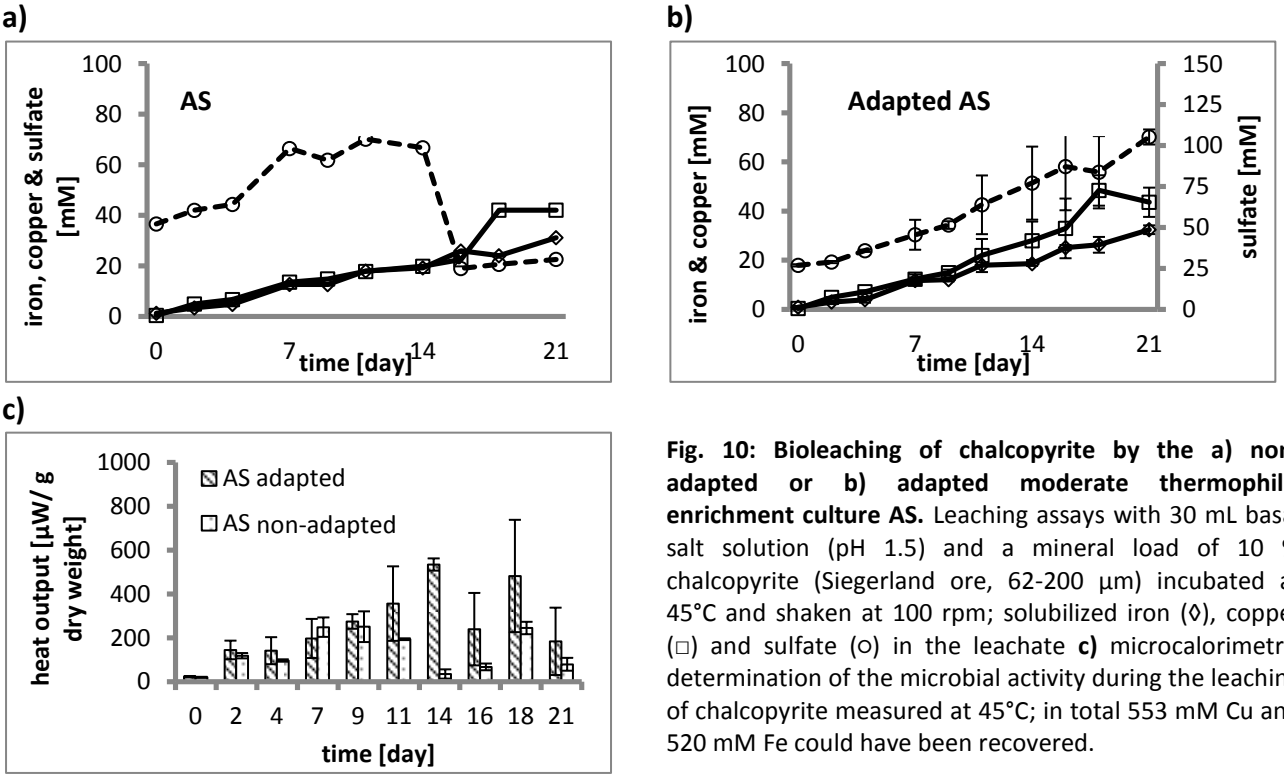

Fig. 10: Bioleaching of chalcopyrite by the a) non-adapted or b) adapted moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 45°C and shaken at 100 rpm; solubilized iron (◊), copper (□) and sulfate (○) in the leachate **c)** microcalorimetric determination of the microbial activity during the leaching of chalcopyrite measured at 45°C; in total 553 mM Cu and 520 mM Fe could have been recovered.

In both assays (adapted or not) pH and cell counts increased with time in a similar way (Fig. 54, 55 and 56, appendix).
The non-adapted culture leached 7.3 % copper, while the adapted one leached 9 %. Both assays leached 7.5 % iron. The sterile control assay leached 2.7 % copper and 1.5 % iron (Fig. 56, appendix).
Calculating the thermal output (considering the copper oxidation rate during the three weeks of leaching), 43 % of the standard reaction energy for the adapted and 50 % for the non-adapted AS assays were detected (Tab. 18).

Tab. 18: Copper oxidation rate and release of thermal energy during chalcopyrite leaching with the non adapted or adapted moderate thermophilic enrichment culture AS.

Cultures	Heat output [µW/ d]	Thermal energy of heat output [J/ g*d]	Copper oxidation rate [mmol/ g*d]	Ratio calculated to standard reaction energy
AS (non adapted)	200	17.28	0.023	**0.43**
AS (adapted)	300	25.92	0.03	**0.50**

4.5 Calorimetric measurements on chalcopyrite degradation at 65°C

Experiments in the thermophilic temperature range were conducted with the archaeon *Sulfolobus metallicus*, shown in Fig. 11.

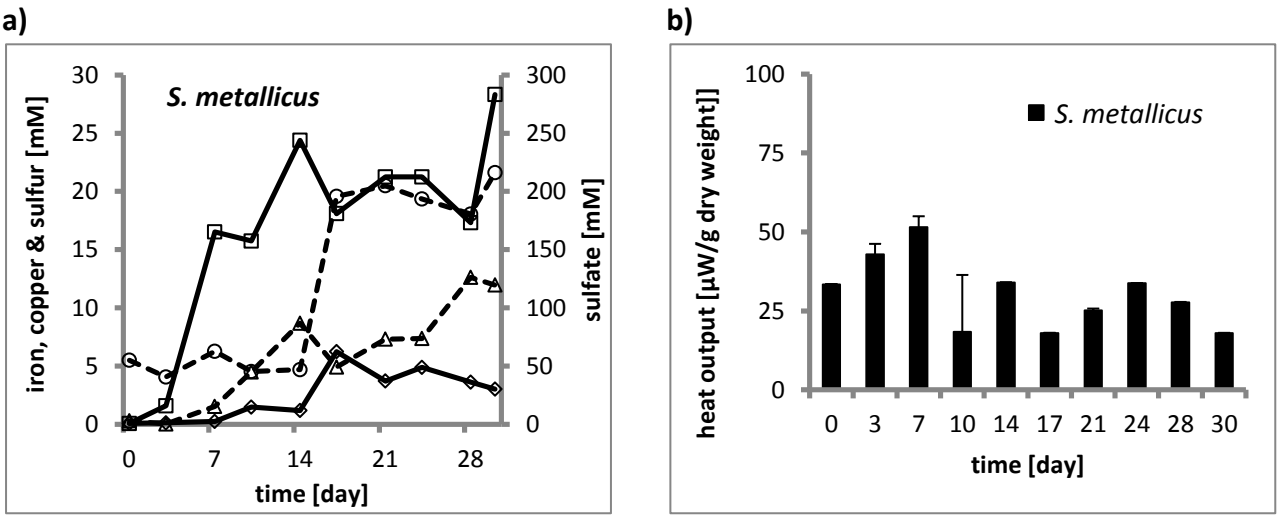

Fig. 11: Bioleaching of chalcopyrite by *S. metallicus*. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 65°C and shaken at 100 rpm; solubilized iron (◊), copper (□), sulfate (○) and sulfur (Δ) in the leachate of *S. metallicus* assays; **b)** microcalorimetric determination of the microbial activity during the leaching of chalcopyrite measured at 65°C; in total 553 mM Cu and 520 mM Fe could have been recovered.

After a decrease of the cell counts in the first week to a concentration of below 10^7 cells/mL, the cell counts increased to almost 10^8 cells/ mL during the following 2 weeks. The pH in the inoculated and the sterile assays increased to values of between 1.9 and 2.1. (Fig. 57, appendix).
The *S. metallicus* assay recovered 5.5 % copper and 1.1 % iron, while the sterile control recovered 3.6 % copper and 0.5 % iron (Fig. 58, appendix). Also considerable amounts of sulfur were produced during the leaching of chalcopyrite by *S. metallicus* (Fig. 11).
Considering the copper oxidation rate from the second week on, and taking into account that there was a significant amount of sulfur produced, 85 % of the standard reaction energy could be detected (Tab. 19).

Tab. 19: Copper oxidation rate and release of thermal energy during chalcopyrite leaching with *S. metallicus*.

Cultures	Heat output [µW/d]	Thermal energy of heat output [J/ g*d]	Copper oxidation rate [mmol/ g*d]	Ratio calculated to standard reaction energy
S. metallicus	30	2.592	0.006	**0.85**

Summarizing the leaching experiments with chalcopyrite, at mesophilic conditions up to 78 % and at thermophilic conditions about 85 % of the standard reaction energy could be detected via microcalorimetry. During moderate thermophilic bioleaching with adapted AS cultures the calorimetric measurements on the degradation process referred to complete dissolution of the mineral.

4.6 Calorimetric measurements of chalcocite and covellite degradation

Besides chalcopyrite bioleaching also chalcocite and covellite bioleaching were investigated. The experiments showed that the pH during chalcocite leaching increased to a value of 4 during the first days of leaching. The pH increase and the increased copper concentration led to a decrease of the cell counts. For covellite leaching no cell growth could be detected.
Experiments with pH adjustment during chalcocite or covellite bioleaching in the mesophilic and moderate thermophilic temperature range were carried out (Fig. 59, 60 and 61, appendix). During mesophilic chalcocite bioleaching not more than 10 % copper could be recovered after 7 weeks, regardless whether the pH had been adjusted or not. In chemical control assays 2 % copper were recovered. With mesophilic covellite bioleaching 7.7 % copper was recovered by pH uncontrolled assays, whereas in pH controlled assays 10 % copper were found. Sterile controls leached 1.3 %. After a lag phase of 14 days cell growth could be detected in all covellite bioleaching assays.
During moderate thermophilic chalcocite bioleaching pH adjustment led to differences in copper recovery for assays inoculated with the enrichment culture AS. Sterile assays without pH adjustment leached 3.4 %, whereas in pH controlled ones 6.8 % were detected. In controlled and with cells of AS inoculated assays 10 % copper were found. Cell counts in all inoculated assays decreased (after 3 weeks below the detection limit of a Thoma chamber, < 10^6 cells/mL).
During the leaching of covellite under moderate thermophilic conditions (regardless whether pH was adjusted or not) the AS inoculated assays could recover 15.5 % copper, while *Sb.*

thermosulfidooxidans inoculated ones recovered the same quantities as the sterile controls (i.e. 1.3 %). Cell growth could be detected for the AS enrichment after a lag phase of one week, which was contrary to *Sb. thermosulfidooxidans*, where cell counts decreased after three weeks below detection limits of a Thoma chamber (< 10^6 cells/ mL).

Further experiments were conducted in order to acquire the heat output during chalcocite and covellite bioleaching. Fig. 12 shows chemical leaching and bioleaching of chalcocite under mesophilic conditions by *At. ferrooxidans* or the enrichments M6 or RAM.

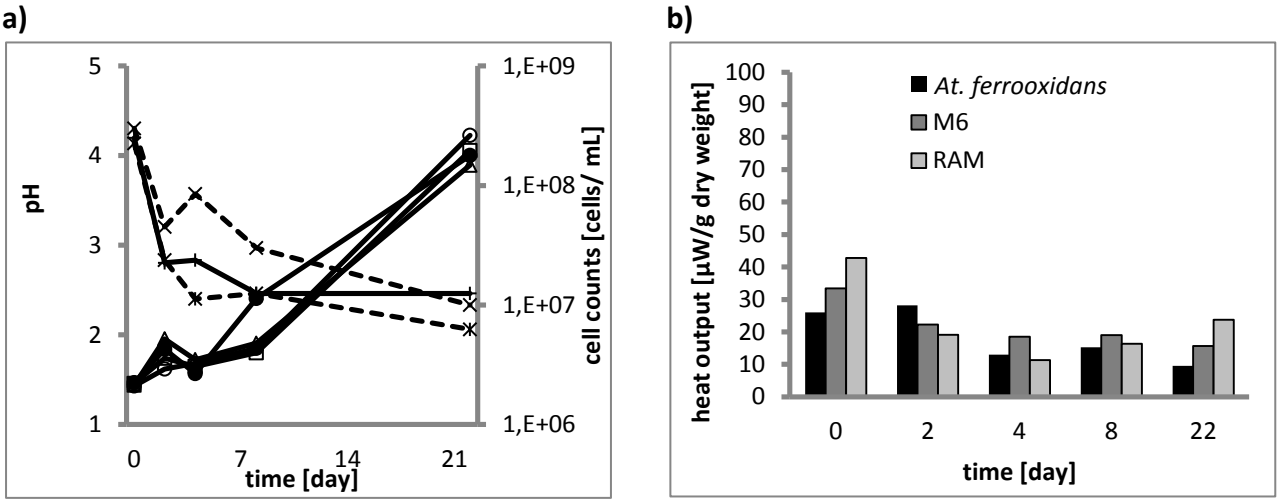

Fig. 12: Chemical leaching and bioleaching of chalcocite by *At. ferrooxidans* or the mesophilic enrichment cultures M6 or RAM. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcocite (62-200 µm) incubated at 28°C and shaken at 100 rpm; **a)** pH of *At. ferrooxidans* assays (○), M6 assays (□), RAM assays (Δ) and chemical control assays (●), cell counts of *At. ferrooxidans* assays (x), M6 assays (ж) and RAM assays (+); **b)** microcalorimetric determination of the microbial activity during the leaching of chalcocite measured at 28°C; in total 737 mM Cu for chalcocite and 802 mM Cu for covellite could have been recovered.

In all assays 125 mM copper were dissolved, this means 17 % copper recovery after three weeks (Fig. 62, appendix). Cell counts were decreasing, while pH was increasing during the experiments. Considering a constant heat output of 20 µW from day 2 to day 8 for all inoculated assays and a copper oxidation rate of 0.05 mmol/g*d, 12 % of the reaction energy of chalcocite dissolution could be rediscovered. During chemical leaching of chalcocite a higher heat output (20%-50%) could be measured in comparison to bioleaching assays.

In Fig. 13 chemical leaching and bioleaching of covellite under mesophilic conditions by *At. ferrooxidans* or the enrichments M6 or RAM are shown.
In all assays the pH increased during the first 4 days and then decreased. Cell counts were decreasing from 5*10^8 cells/mL to below 5*10^7 cells/mL. During three weeks of chemical or microbial leaching of covellite 9 % of copper could be recovered in all assays (Fig. 63, appendix).
Microcalorimetrically determined heat output values were similar for inoculated and sterile assays. Covellite can be dissolved by ferric iron. Considering a constant heat output of 10 µW from day 2 to

day 8 for all inoculated assays and a copper oxidation rate of 0.016 mmol/g*d, 15 % of the standard reaction energy of covellite dissolution could be detected.
A repetition of the experiment with prior pH adjustment (before inoculation) showed similar results (Fig. 64, appendix).

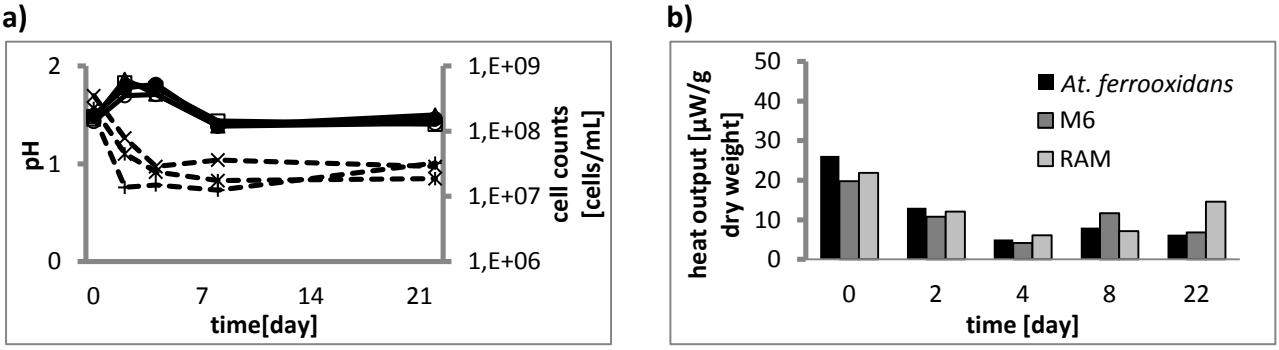

Fig. 13: Chemical leaching and bioleaching of covellite by *At. ferrooxidans* or the mesophilic enrichment cultures M6 or RAM. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % covellite (62-200 µm) incubated at 28°C and shaken at 100 rpm; **a)** pH of *At. ferrooxidans* assays (○), M6 assays (□), RAM assays (Δ) and chemical control assays (●), cell counts of *At. ferrooxidans* assays (x), M6 assays (ж) and RAM assays (+) **b)** microcalorimetric determination of the microbial activity during the leaching of covellite measured at 28°C; in total 802 mM Cu could have been recovered.

In Fig. 14, the chemical leaching and microbial leaching of chalcocite under moderate thermophilic conditions with the enrichment culture AS are shown.

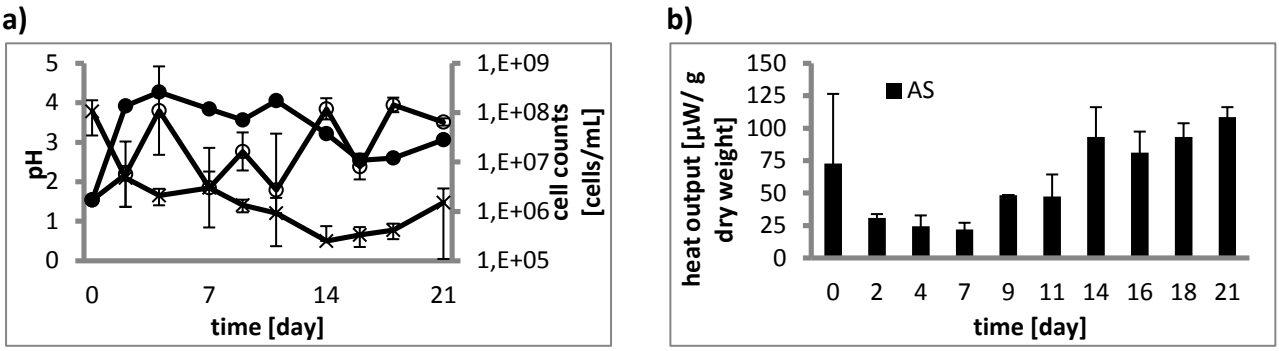

Fig. 14: Chemical leaching and bioleaching of chalcocite at 45°C by the moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcocite (62-200 µm) incubated at 45°C and shaken at 100 rpm; **a)** pH of AS assays (○), pH of chemical control assays (●) and cell counts of AS assays (x); **b)** microcalorimetric determination of the microbial activity during the leaching of chalcocite measured with at 45°C; in total 737 mM Cu could have been recovered.

During the leaching of covellite at 45 °C a total copper recovery of 17 % after 21 days was measured for the sterile and inoculated assays (Fig. 65, appendix). Cell counts decreased and pH increased during the experiment. Sterile control assays gave a higher heat output signal than the ones inoculated with the enrichment culture AS. While in the chemical control 43 % of the standard reaction energy of covellite dissolution were detectable during three weeks of leaching, only 32 % were gathered for the AS assays.

Figure 15 shows covellite leaching at 45 °C by the moderate thermophilic enrichment culture AS. In all assays 8 % copper were recovered (Fig. 66 and 67, appendix).

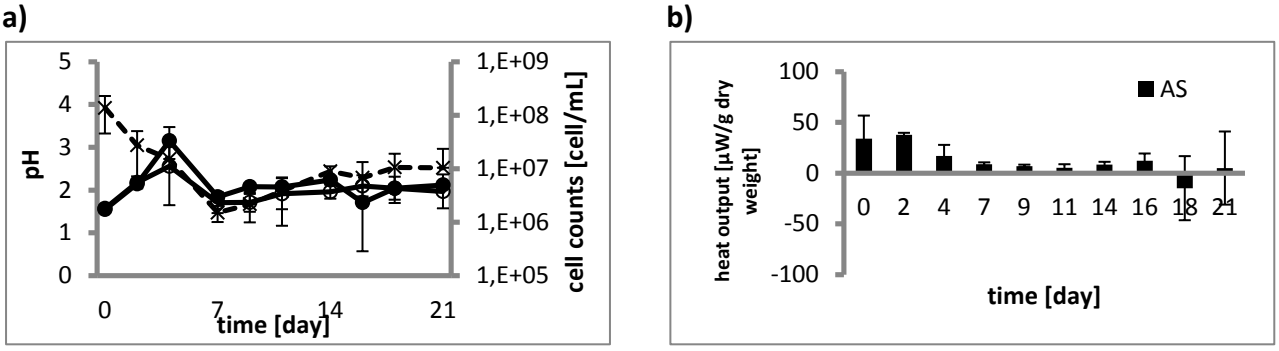

Fig. 15: Chemical leaching and bioleaching of covellite at 45°C by the moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % covellite (62-200 µm) incubated at 45°C and shaken at 100 rpm; **a)** pH of AS assays (○), pH of chemical control assays (●) and cell counts of AS assays (x); **b)** microcalorimetric determination of the microbial activity during the leaching of covellite measured at 45°C; in total 802 mM Cu could have been recovered.

During the first 4 days of leaching a thermal output equivalent to 11 % of the standard reaction energy could be detected. In order to determine how pH adjustment influences the acid consumption of the minerals chalcocite and covellite further experiments were conducted (Fig. 16).

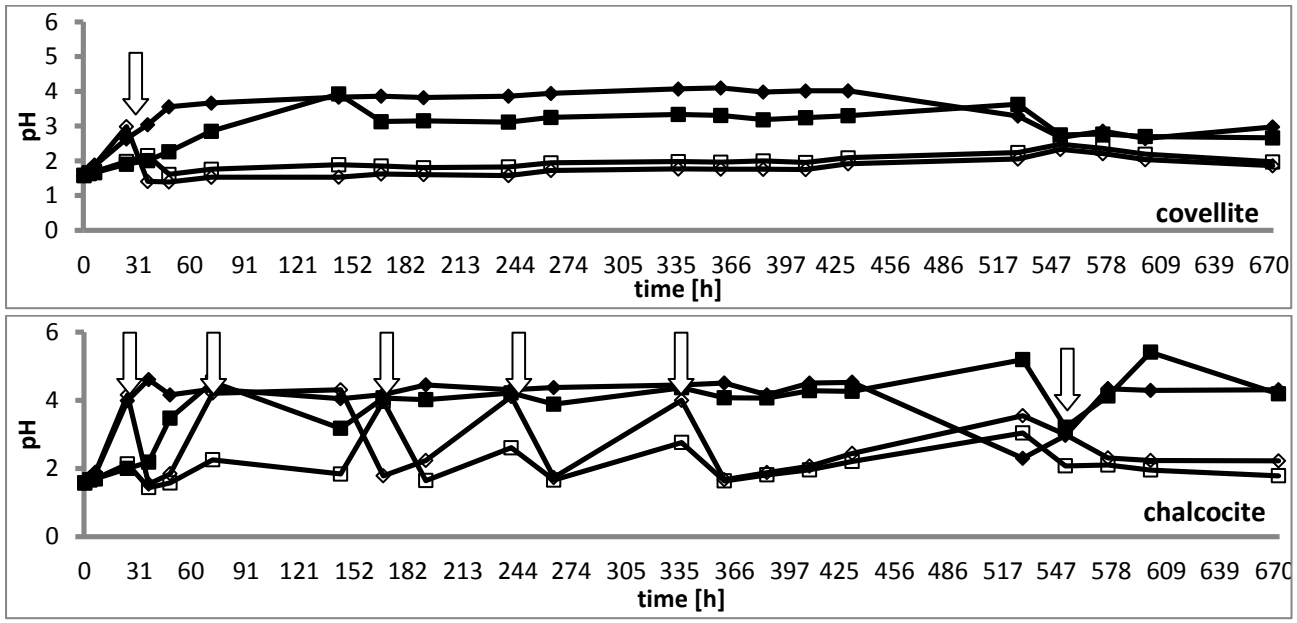

Fig. 16: Evolution of the pH in sterile assays leaching covellite or chalcocite at 28°C or 45°C with or without pH adjustment. Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 10% mineral (62-200 µm) incubated at 28°C or 45°C shaken at 120 rpm; pH was adjusted to below 2 with conc. sulfuric acid; assays incubated at 28°C with (◊) and without pH adjustment (♦); assays incubated at 45°C with (□) or without pH adjustment (■); arrows indicate addition of sulfuric acid for pH adjustment.

During covellite leaching pH adjustment directly after leaching start was sufficient to keep the pH below a value of 2 over the whole test period. To prevent pH increase during chalcocite leaching the pH has to be adjusted at least every hour. The acid consumption in the mineral assays at 28 °C or 45 °C was similar.

4.7 Microscopic observations during chalcopyrite bioleaching

To observe the biofilm development during the first two weeks of chalcopyrite bioleaching, confocal laser microscopic images were taken. For this purpose, mesophiles like *At. ferrooxidans* and the enrichments M6 and RAM were prepared, next to assays with moderate thermophiles like *At. caldus* and the enrichment AS. The microorganisms were pre-cultivated on different substrates prior to inoculation of the assays. In Fig. 17 CLSM images of *At. ferrooxidans* attached to chalcopyrite are shown. The cells were pre-cultivated on sulfur, iron sulfate, pyrite or chalcopyrite.

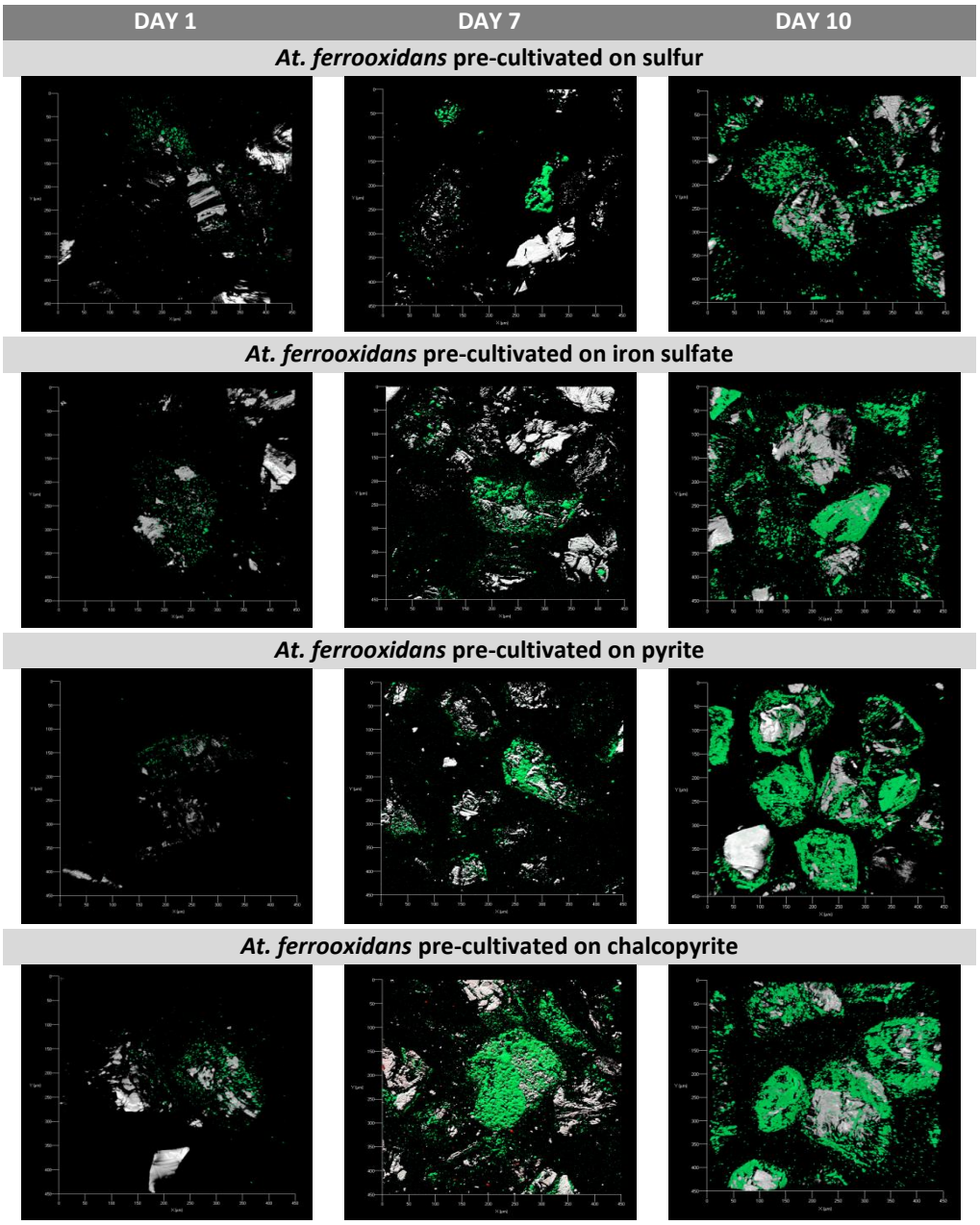

Fig. 17: Attachment of *At. ferrooxidans* to chalcopyrite visualized by CLSM. Cells were pre-cultivated on sulfur, iron sulfate, pyrite or chalcopyrite and then incubated with 1 % chalcopyrite in 50 mL basal salt solution (pH 1.5) at 28°C and shaken at 100 rpm. Samples were taken after 1, 7 and 10 days and stained by Syto®9 (green).

A high cell attachment was observed at day 10 for sulfur, iron sulfate and pyrite grown cells. For chalcopyrite grown cells a comparable attachment pattern was detectable at day 7.

Pyrite and chalcopyrite grown cells showed the highest coverage of the chalcopyrite surface, while iron sulfate and especially sulfur grown cells showed less surface coverage.
Although the attachment patterns were different concerning the pre-cultivation conditions, the copper and iron recoveries were similar for all assays (Fig. 68 a and b, appendix). The iron recovery ranges from 0.9 % to 1.6 % and approximately 1 % copper was recovered.

Fig. 18 shows the CLSM images of the mesophilic enrichment culture M6 pre-cultivated on iron sulfate, pyrite or chalcopyrite for bioleaching of chalcopyrite.

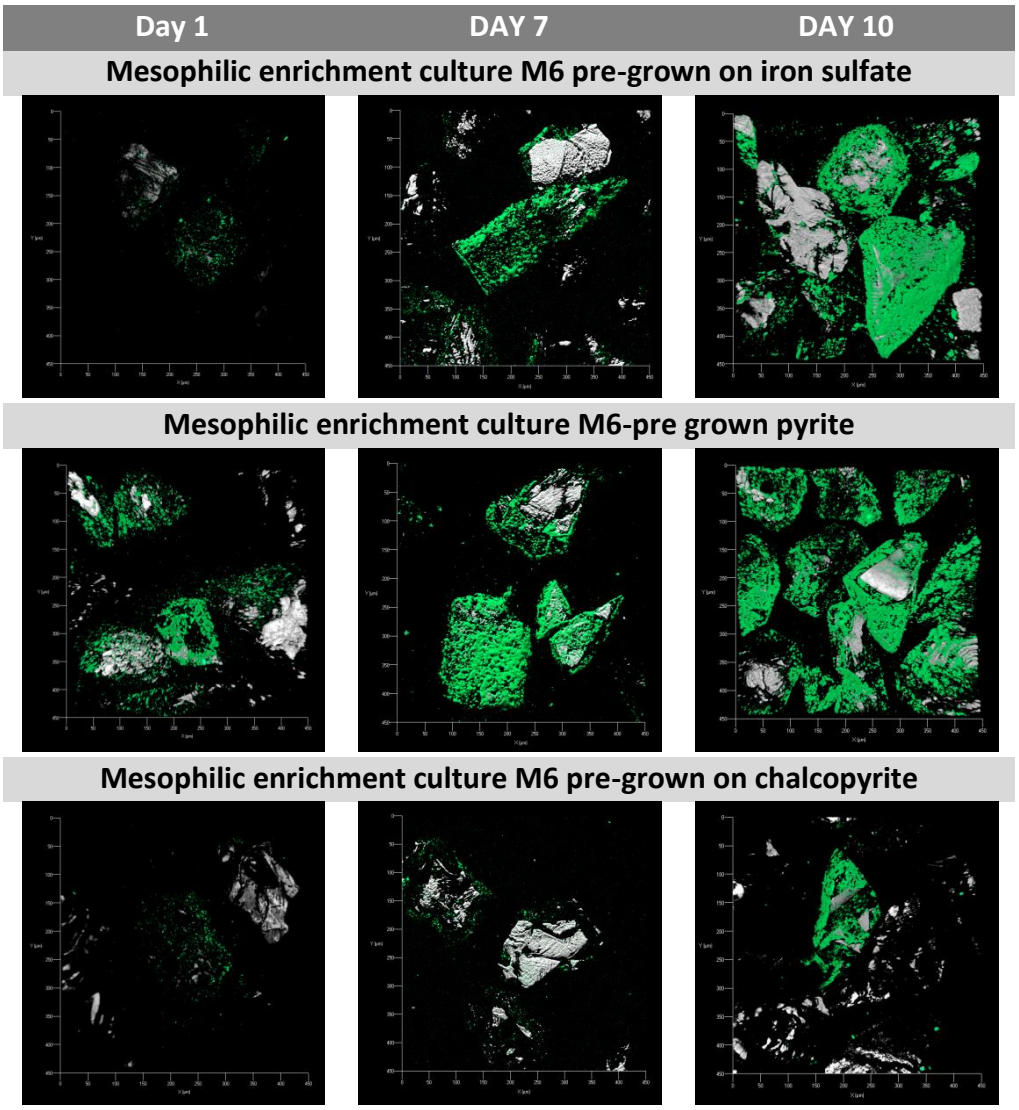

Fig. 18: Attachment of the mesophilic enrichment culture M6 to chalcopyrite visualized by CLSM. Cells were pre-cultivated on iron sulfate, pyrite or chalcopyrite and then incubated with 1 % chalcopyrite in 50 mL basal salt solution (pH 1.5) at 28°C and shaken at 100 rpm. Samples were taken after 1, 7 and 10 days and stained by Syto®9 (green).

A high surface coverage of chalcopyrite by pyrite pre-grown cells of the mesophilic enrichment culture M6 could be seen from day 1 onwards. Iron sulfate grown cells showed high attachment after the first week. Compared to the cells pre-cultivated with iron sulfate or pyrite, cells grown with chalcopyrite showed less coverage of the chalcopyrite surface during the first two weeks.
The iron and copper recoveries (Fig. 68 c and d, appendix) were similar to the ones achieved by *At. ferrooxidans*, i.e. 1 % to 1.5 % or approximately 1 %, respectively.

In Fig. 19 CLSM images of the chalcopyrite leaching mesophilic enrichment culture RAM pre-cultivated on iron sulfate, pyrite or chalcopyrite are shown.

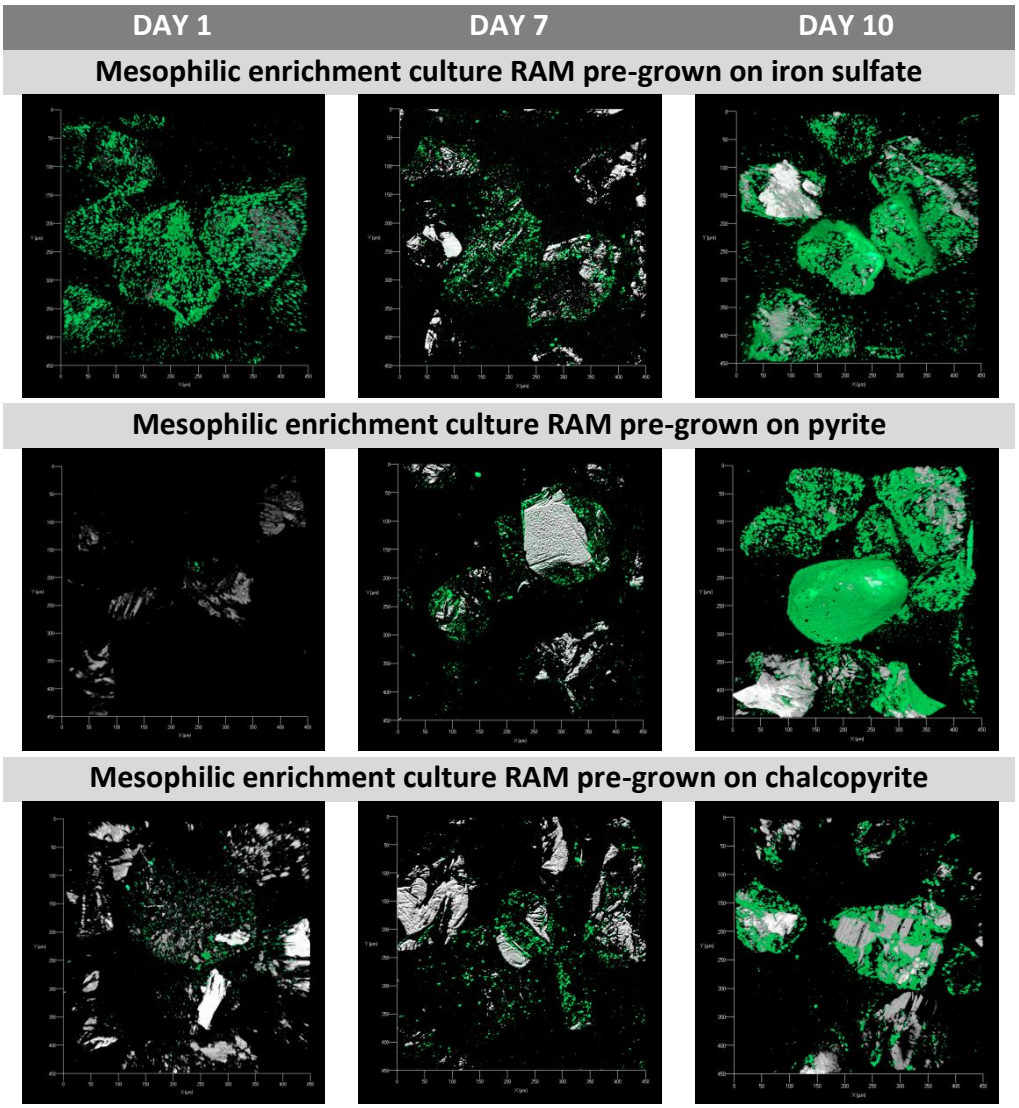

Fig. 19: Attachment of the mesophilic enrichment culture RAM to chalcopyrite visualized by CLSM. Cells were pre-cultivated on iron sulfate, pyrite or chalcopyrite and then incubated with 1 % chalcopyrite in 50 mL basal salt solution (pH 1.5) at 28°C and shaken at 100 rpm. Samples were taken after 1, 7 and 10 days and stained by Syto®9 (green).

A high surface coverage of chalcopyrite by microbial cells could be seen after day 1 with the mesophilic enrichment culture RAM pre-grown on iron sulfate. After 10 days a high surface coverage was visible for assays with pyrite pre-grown cells. Cells pre-grown on chalcopyrite showed less attachment to chalcopyrite compared to the others.
Iron recoveries ranged from 0 % to 1.6 % and copper recoveries from 3.1 % to 6.2 % (Fig. 68 e and f, appendix). Sterile controls leached 0.8 % iron and 1 % copper from chalcopyrite (Fig. 68 g, appendix).

In Fig. 20 the CLSM images of chalcopyrite leaching *At. caldus* cultures pre-grown on sulfur or chalcopyrite are shown.

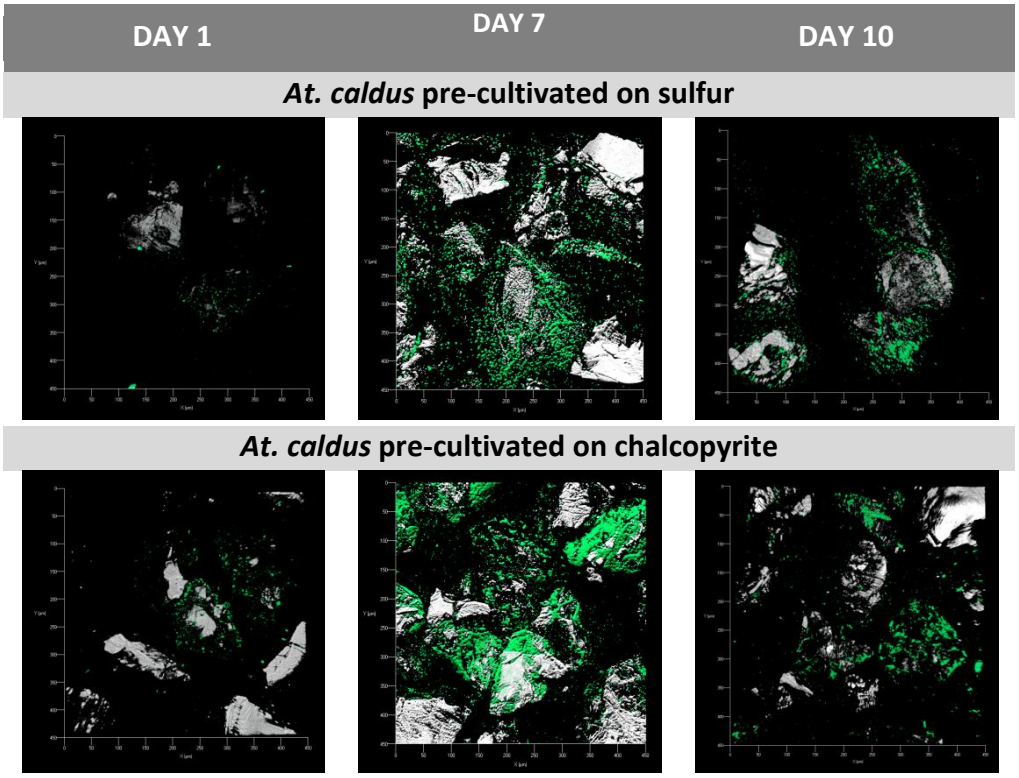

Fig. 20: Attachment of *At. caldus* to chalcopyrite visualized by CLSM. Cells were pre-cultivated on sulfur or chalcopyrite and then incubated with 1 % chalcopyrite in 50 mL basal salt solution (pH 1.5) at 45°C and shaken at 100 rpm. Samples were taken after 1, 7 and 10 days and stained by Syto®9.

Compared to the attachment patterns presented before, *At. caldus* showed less attachment to chalcopyrite. Sulfur pre-grown cells showed high detachment after day 14. However, 1 % to 1.8 % iron and 3.6 % to 6.8 % copper could be recovered during bioleaching with *At. caldus* (Fig. 69 a and b, appendix).

Fig. 21 shows the CLSM images of the chalcopyrite leaching moderate thermophilic enrichment culture AS pre-cultivated on sulfur, pyrite or chalcopyrite. Cells pre-cultivated on pyrite or chalcopyrite showed high surface coverage after one week of chalcopyrite bioleaching, while cells grown on sulfur show high detachment on day 10. Iron recoveries ranged from 1 % to 1.5 % and

copper recoveries from 6 % to 6.6 % (Fig. 69 c and d, appendix). Iron and copper recoveries of the sterile control at 45 °C were 1.6 % iron and 1.7 % copper (Fig. 69 g).

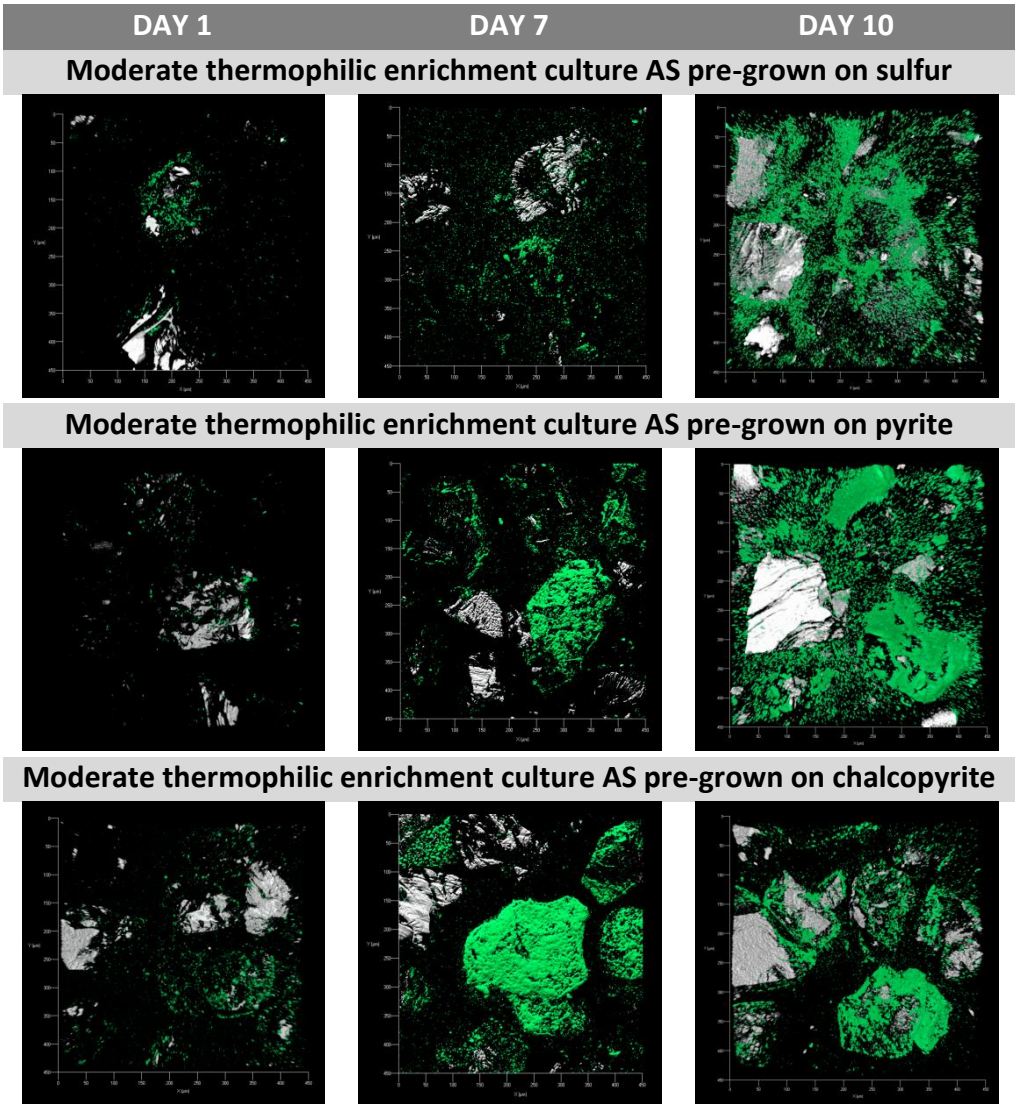

Fig. 21: Attachment of the moderate thermophilic enrichment culture AS to chalcopyrite visualized by CLSM. Cells were pre-cultivated on sulfur, pyrite or chalcopyrite and then incubated with 1 % chalcopyrite in 50 mL basal salt solution (pH 1.5) at 45°C and shaken at 100 rpm. Samples were taken after 1, 7 and 10 days and stained by Syto®9 (green).

Summarizing, a high microbial population density on chalcopyrite did not result in advanced leaching and/or increased copper (or iron) recovery. Thermophilic bioleaching dissolves more copper than mesophilic bioleaching. Cells pre-cultivated on sulfur were easily detached from chalcopyrite surfaces.

4.8 Cuprous copper and copper sulfide bioleaching

In addition to the AAS measurements copper was determined by a photometric method according to Anwar *et al.* (2000). This procedure allows to distinguish between the oxidation state, cuprous or cupric copper.

Evolution of cuprous copper during copper sulfide leaching. Fig. 22 shows the evolution of solubilized copper and iron species during the dissolution of chalcopyrite by the moderate thermophilic enrichment culture AS or in a sterile control assay at 45 °C.

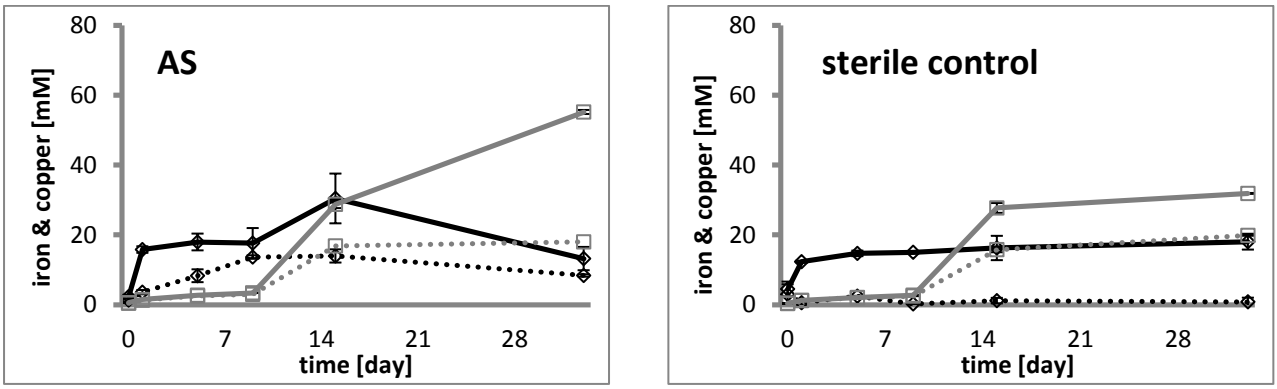

Fig. 22: Occurrence of copper and iron speciation during bioleaching of chalcopyrite by the moderate thermophilic enrichment culture AS or in a sterile control assay. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 45°C and shaken at 100 rpm; total iron (black solid line ◊), ferric iron (black dashed line ◊) and total copper (gray solid line □), cuprous copper (gray dashed line □); in total 553 mM Cu and 520 mM Fe could have been recovered; in total 553 mM Cu and 520 mM Fe could have been recovered.

During the first two weeks, the cell counts decreased from 10^8 to $5*10^6$ cells/ mL, afterwards the cell density increased up to 10^7 cells/ mL. The pH value of the sterile and inoculated assay increased during the first week up to 2 (Fig. 70 a, appendix). The leaching of chalcopyrite by the moderate thermophilic enrichment culture AS could recover 9 % copper, while the sterile assay leached 5.5 %. In the sterile assay 3.5 % iron could be solubilized, the moderate thermophilic enrichment culture could solubilize 6 % iron during the first two weeks. However, the iron recovery in the inoculated assay decreased after two weeks to 3 % (Fig. 70 b and c).
Regarding the iron species, during the chemical leaching of chalcopyrite only ferrous iron could be measured, while during bioleaching with the moderate thermophilic enrichment AS most of the iron was present as ferric iron (Fig. 22). Two thirds of the solubilized copper amount in the control assay was present as cuprous copper, one third as cupric copper. In the assay with the bacteria approximately 40 % of the total copper was present as cuprous copper (Fig. 22).

The copper and iron speciations during the leaching of pyrite containing copper sufides (Fig. 23), chalcopyrite (Fig. 24) or covellite (Fig. 25) were measured and evaluated in the mesophilic or moderate thermophilic temperature range by *At. ferrooxidans* or the moderate thermophilic enrichment culture AS.

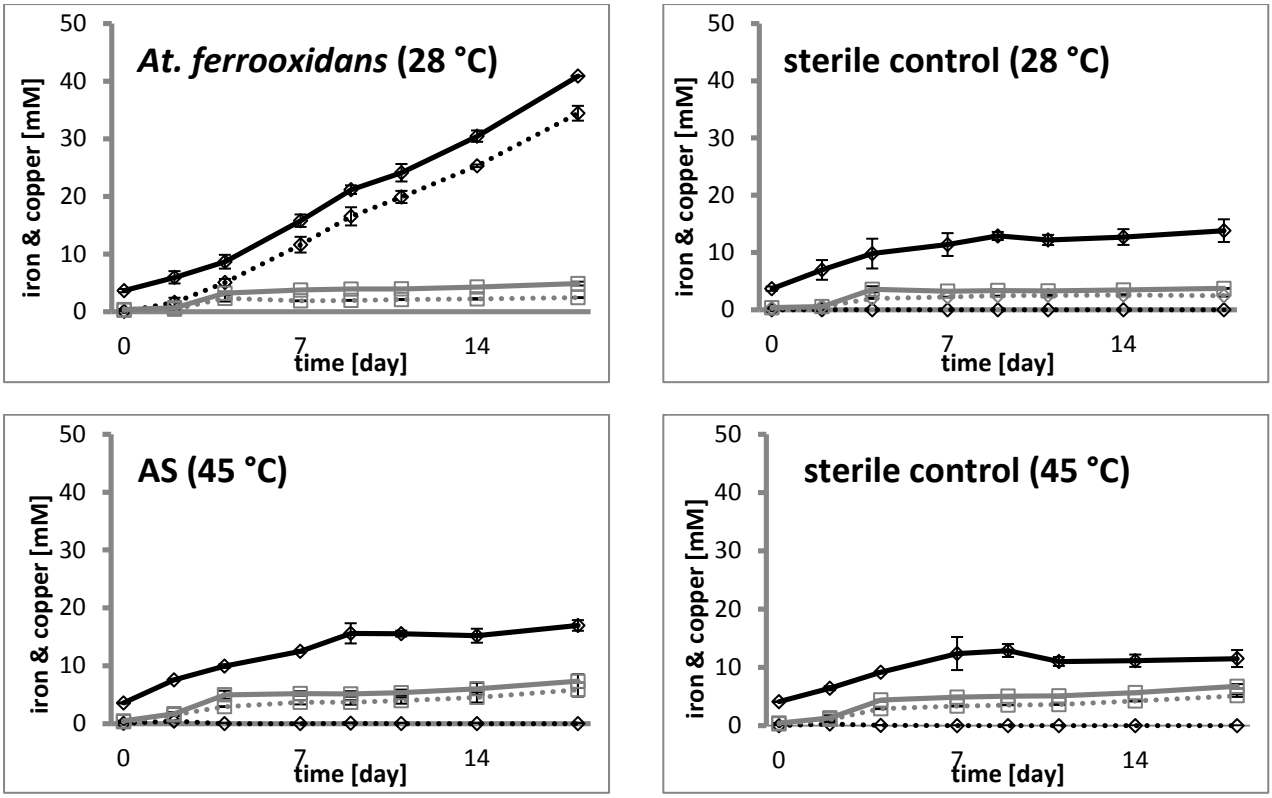

Fig. 23: Copper and iron speciation arising during bioleaching of pyrite containing copper sulfides by *At. ferrooxidans* at 28°C or the moderate thermophilic enrichment culture AS at 45°C. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % pyrite containing copper sulfides (Romania ore, 62-200 µm) incubated and shaken at 100 rpm; total iron (black solid line ◊), ferric iron (black dashed line ◊) and total copper (gray solid line □), cuprous copper (gray dashed line □); in total 553 mM Cu and 520 mM Fe could have been recovered; in total 553 mM Cu and 520 mM Fe could have been recovered.

The cell count of *At. ferrooxidans* during the leaching assays with pyrite containing copper sulfides increased from 10^8 to 10^9 cells/mL, while the cell counts of the moderate thermophilic enrichment culture AS decreased to $5*10^7$ cells/mL during the experiments (Fig. 71 a and 72 a, appendix). The pH values of all assays varied between 1.5 and 2 (Fig. 71 a and 72 a, appendix).

The sterile assays at 28 °C and 45 °C leached 10 mM iron (8.5 %) and 5 mM copper (17 %; Fig. 71 c and 72 c, appendix). The same amount of iron was solubilized during the bioleaching of pyrite containing copper sulfides by the mesophilic culture of *At. ferrooxidans* and the moderate thermophilic enrichment culture AS. During the bioleaching of pyrite containing copper sulfides by *At. ferrooxidans* 34 % copper could be recovered, while by the moderate thermophilic enrichment culture AS 15 % copper was recovered (Fig. 71 b and 72 b, appendix).

Fig. 23 shows cases, where no (or only few ferric iron) could be detected. Solubilized iron was present as ferrous iron and almost all copper was present as cuprous copper (Fig. 23 b, c and d).

In the *At. ferrooxidans* assay 90 % of the total iron could be measured as ferric iron and about half of the measured copper was present as cuprous copper (Fig. 23 a).

In Fig. 24 the copper speciation arising during the leaching of chalcopyrite by *At. ferrooxidans* or the moderate thermophilic enrichment culture AS are shown.

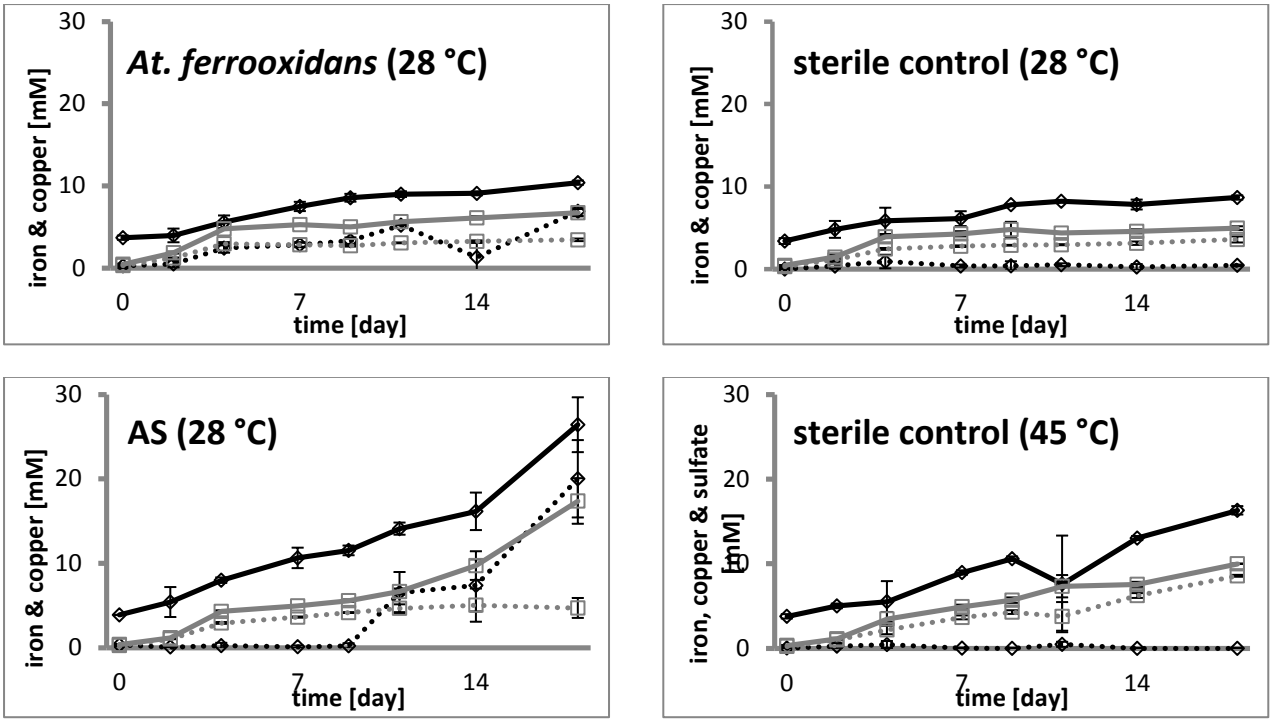

Fig. 24: Copper and iron speciation arising during bioleaching of chalcopyrite by *At. ferrooxidans* at (28°C) or the moderate thermophilic enrichment culture AS at (45°C). Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated and shaken at 100 rpm; total iron (black solid line ◊), ferric iron (black dashed line ◊) and total copper (gray solid line □), cuprous copper (gray dashed line □); in total 553 mM Cu and 520 mM Fe could have been recovered; in total 553 mM Cu and 520 mM Fe could have been recovered.

The cell counts of *At. ferrooxidans* and the moderate thermopilic enrichment culture AS remained constant during the experiment (Fig. 73 a and 74 a, appendix). The pH of the sterile assay at 28 °C increased up to a value of 3, while the pH of the *At. ferrooxidans* assay increased during the first week and then decreased. In the assays at 45 °C the pH increased up to a value of 3, too. However, the pH of the AS assay decreased after two weeks to 1.5 (Fig. 73 a and 74 a, appendix).

The sterile assays at 28 °C and 45 °C leached 10 mM iron (2 %) and 10 mM copper (2 %; Fig. 73 c and 74 c, appendix). The same amount of iron was solubilized during the bioleaching of pyrite containing copper sulfides by the mesophilic culture of *At. ferrooxidans* or the moderate thermophilic enrichment culture AS. During the leaching with the moderate thermophilic enrichment culture AS 15 mM (3 %) copper and 30 mM (6 %) iron could be recovered (Fig. 74 b, appendix).

In both sterile assays (28 °C and 45 °C) no ferric iron could be measured and 80 % of the copper measured was detected as cuprous copper (Fig. 24 b and d). While the bioleaching of chalcopyrite by *At. ferrooxidans* half of the iron measured was detected as ferric iron and 60 % of the copper as cuprous copper (Fig. 24 a). Meanwhile, in the first ten days of chalcopyrite bioleaching with the moderate thermophilic enrichment culture AS no ferric iron could be detected and 80 % of the copper was measured as cuprous copper. However, after ten days the ferric iron level increased constantly and the cuprous copper level remained stable while the total copper concentration increased (Fig. 24 c).

In Fig. 25 the copper speciation during the leaching of covellite by *At. ferrooxidans* or the moderate thermophilic enrichment culture AS are shown.

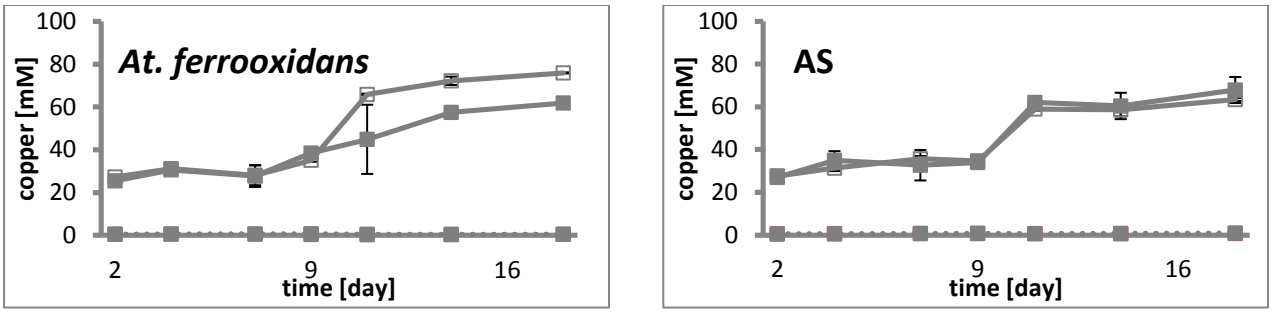

Fig. 25: Copper and iron speciation arising during bioleaching of covellite by *At. ferrooxidans* at (28°C) or the mesophilic enrichment culture AS at (45°C). Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % covellite (62-200 µm) incubated and shaken at 100 rpm; total copper (gray solid line □), cuprous copper (gray dashed line □) in the leachate of inoculated assays and total copper (gray solid line ■), cuprous copper (gray dashed line ■) in the leachate of sterile assays; in total 802 mM Cu could have been recovered.

The cell counts during the bioleaching of covellite remained stable in the *At. ferrooxidans* assays and decreased in the AS assays. The pH values of the sterile assays at both temperatures remained stable during covellite leaching. In the *At ferrooxidans* assays, the pH increased after 10 days to above 3, the same happened in the AS assays after two weeks (Fig. 75 a and 76 a, appendix).
During the leaching of covellite 7,5 % copper could be recovered in the sterile assays and the AS assay, 9 % copper could be recovered in the *At. ferrooxidans* assay (Fig. 75 b and c ,76 b and c, appendix).
Fig. 25 shows that cuprous copper could not be detected during the dissolution of covellite. Also no iron could be detected during the leaching of covellite.

Cuprous copper stability in basal salt solution. To assess the effects of cuprous copper on the activity of bioleaching microorganisms several cuprous copper concentrations and the stability of cuprous copper were tested.
In Fig. 26 the stability of cuprous copper (CuCl dissolved in HCl) in basal salt solution supplemented with sulfur or ferrous iron sulfate is shown.

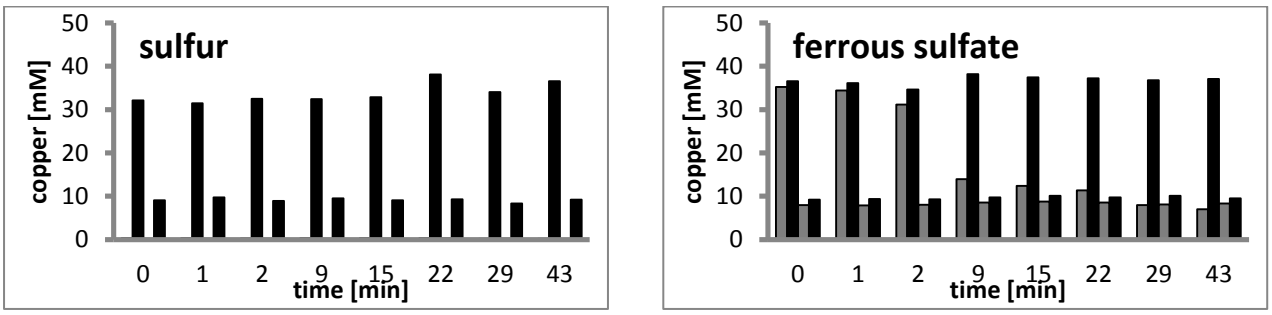

Fig. 26: Total cuprous copper concentration in basal salt solution supplemented with sulfur or ferrous iron sulfate. 50 mL of basal salt solution with 1 g/L sulfur (sulfur flower) or 70 mM ferrous iron incubated at 28 °C and shaken at 100 rpm; supplemented with 10 or 35 mM cuprous copper (CuCl stock was dissolved in 11 M hydrochloric acid); cuprous copper (■) and cupric copper (■).

In basal salt solution supplemented with sulfur and 30 mM or 10 mM cuprous copper no cuprous copper could be measured immediately after the addition (Fig. 26 a). When the basal salt solution was supplemented with ferrous iron sulfate (70 mM), the cuprous copper (30 mM) concentration was stable for 2 min. Afterwards only 10 to 12 mM cuprous copper could be detected in the basal salt solution. While addition of 10 mM cuprous copper, the concentration remained stable for at least 43 minutes (Fig. 26 b). These experiments were conducted at 28 °C (Fig. 26) as well as at 45 °C (data not shown) and showed similar results.

The stability of a cuprous chloride solution (Sigma Aldrich) within basal salt solution supplemented with sulfur or various amounts of ferrous iron sulfate is shown in Fig. 27.

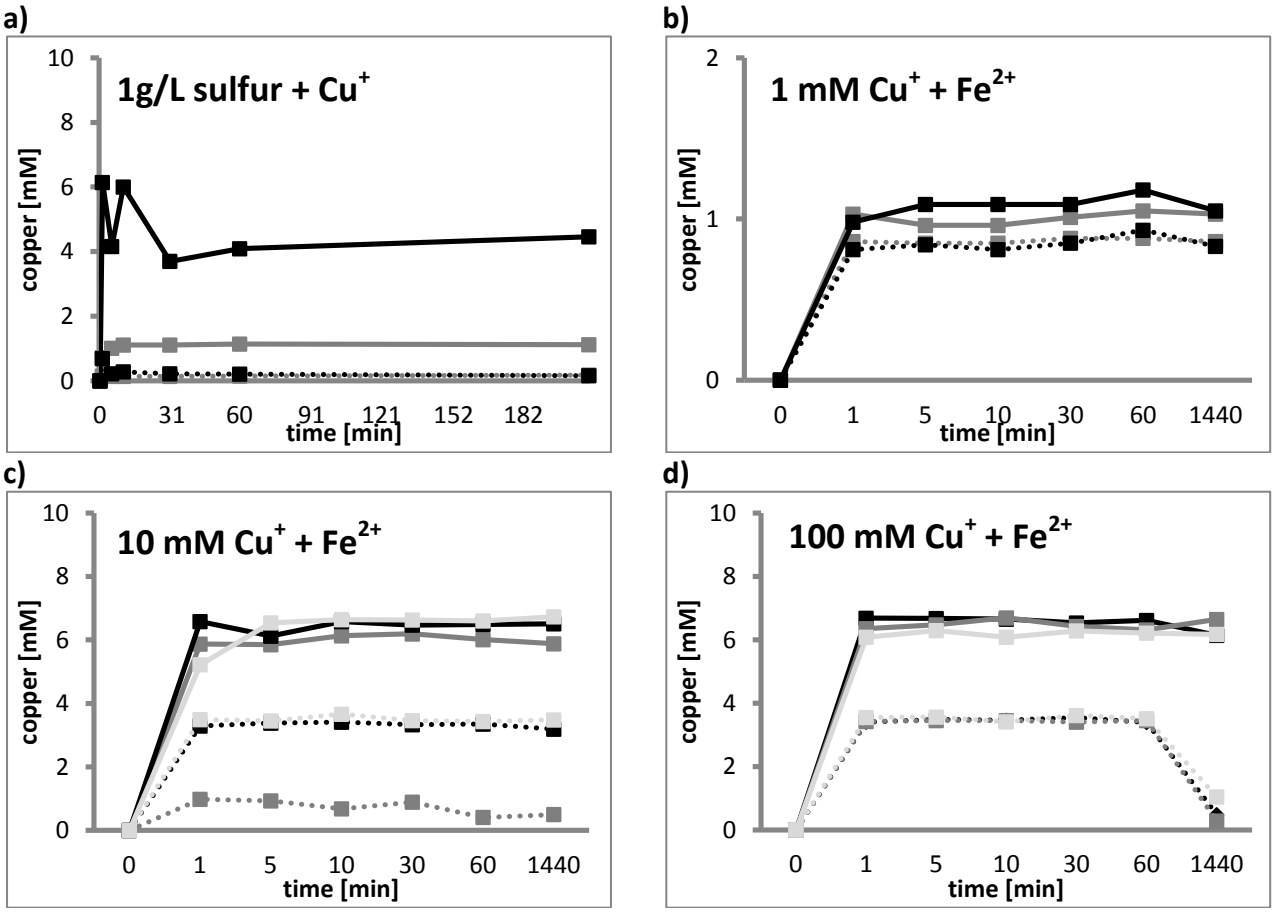

Fig. 27. Stability of 1 mM, 10 mM and 30 mM cuprous copper in basal salt solution supplemented with sulfur or various amounts of ferrous sulfate. a) 50 mL basal salt solution (pH 1.5) supplemented with 1 g/L sulfur (powder), incubated at 28 °C and shaken at 100 rpm, (■) 1 mM and (■) 10 mM cuprous copper, total copper (bold line) and cuprous copper (dashed line); 50 mL basal salt solution supplemented with **b)** 1 mM, **c)** 10 mM and **d)** 100 mM cuprous copper incubated at 28 °C and shaken at 100 rpm, (■) 1 mM ferrous iron, (■) 10 mM ferrous iron and (■) 100 mM ferrous iron, total copper (bold line) and cuprous copper (dashed line).

Cuprous copper could not be measured in basal salt solution supplemented with sulfur immediately after addition of the copper chloride solution (Fig. 27 a). Fig. 27 b shows the concentration of total and cuprous copper upon addition of 1 mM copper chloride in basal salt solution supplemented with 1, 10 or 100 mM ferrous iron sulfate. In all assays most of the copper was present as cuprous

copper. When 10 mM copper chloride solution was added to basal salt solution supplemented with 10 or 100 mM ferrous iron sulfate only half of the total copper measured was present as cuprous copper. With a supplementation of 1 mM ferrous iron sulfate less than 10 % of the total copper measured was present as cuprous copper (Fig. 27 c). If a concentration of 10 mM or more copper chloride solution was added to the basal salt solution, only 7 mM total copper could be measured. During the addition of copper chloride solution (higher than 10 mM) large amounts of precipitation could be observed. When a concentration of 100 mM copper chloride solution was added to basal salt solution supplemented with 1, 10 or 100 mM ferrous iron sulfate, 50 % of the total copper measured was present as cuprous copper. After 24 h no cuprous copper could be measured in the basal salt solution (Fig. 27 d).

Influence of sulfate, ferrous or ferric iron on the cuprous copper stability. Experiments with chalcopyrite (Fig. 28), chalcocite (Fig. 29) and covellite (Fig. 30) were conducted in order to follow the evolution and stability of cuprous copper during the dissolution of these mineral sulfides.
In Fig. 28 the concentration of solubilized iron, copper and sulfate during the dissolution of chalcopyrite with a supplementation of 100 mM sulfate, 50 mM ferrous or ferric iron is shown.

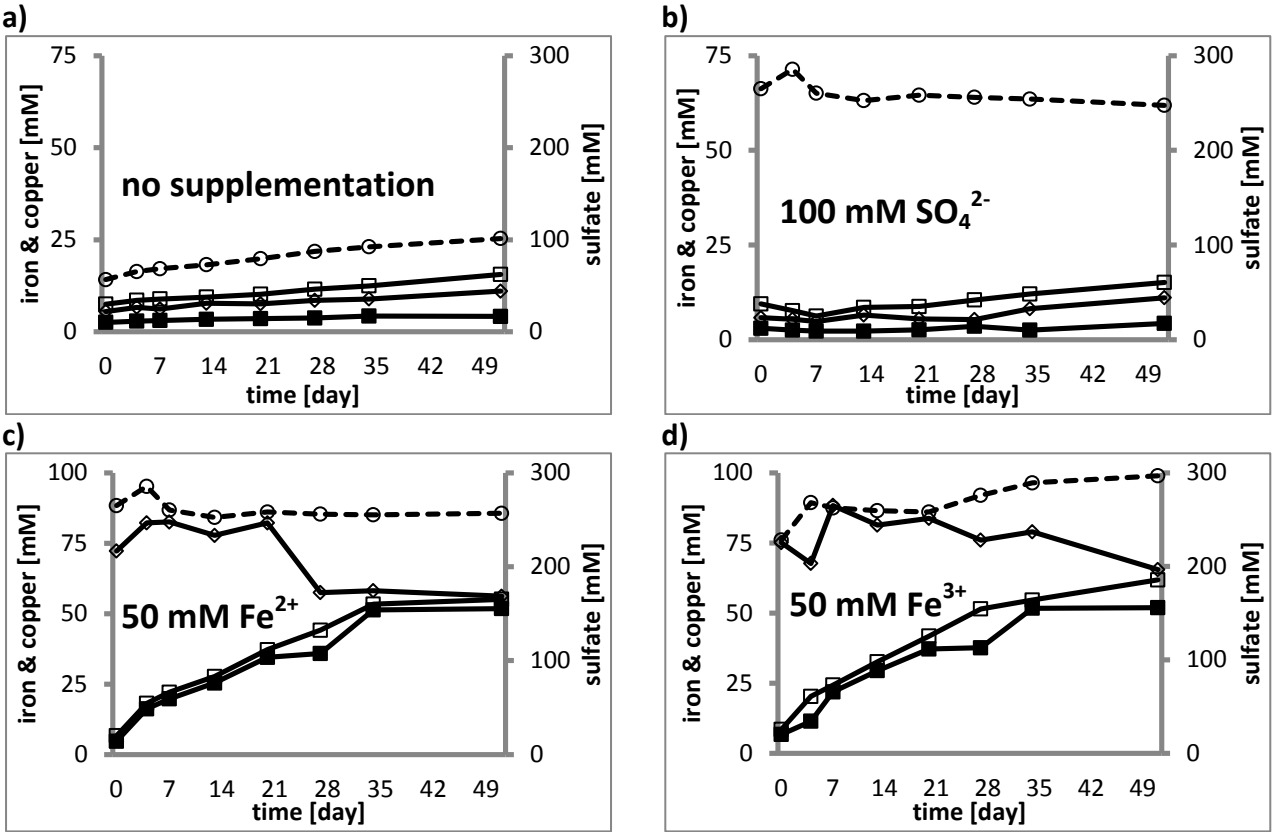

Fig. 28: Solubilized sulfate, iron and copper speciation during chemical leaching of chalcopyrite at 45°C supplemented with sulfate, ferrous or ferric iron. Leaching assays with 30 mL of basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (62-200µm) were incubated at 45°C and shaken at 100 rpm; chalcopyrite leached a) without any supplementation, b) 100 mM sulfate (magnesium sulfate), c) 50 mM ferrous iron (iron(II) sulfate) and d) ferric iron (iron(III) sulfate) supplementation; solubilized iron (◊), copper (□), cuprous copper (■) and sulfate (○) in the leachate; in total 553 mM Cu and 520 mM Fe could have been recovered.

Without supplementation 12 mM copper and 10 mM iron was solubilized after 49 days; 20 % of the total copper measured was present as cuprous copper (Fig. 28 a). The supplementation of 100 mM sulfate showed similar results (Fig. 28 b). Upon the addition of ferrous iron, the concentration of solubilized copper increased up to 50 mM, and 90 % of the copper measured was present as cuprous copper. After 21 days the iron concentration decreased by 25 mM and in parallel no further copper was dissolved (Fig. 28 c). Immediately after the addition of ferric iron sulfate all iron was present as ferrous iron, up to 60 mM copper could be dissolved, 90 % present as cuprous copper. After three weeks the iron concentration decreased by 5 mM (Fig. 28 d). The pH value of the assay without supplementation increased within two weeks to 3. Within the assays supplemented with sulfate or ferrous iron the pH increased to a value of 3 after five weeks. The pH in the assay supplemented with ferric iron increased during the experiment to a value of 2.5 (Fig. 77 a, appendix).

The concentration of solubilized sulfate, iron and copper speciation during the chemical leaching of chalcocite at 45 °C is shown in Fig. 29.

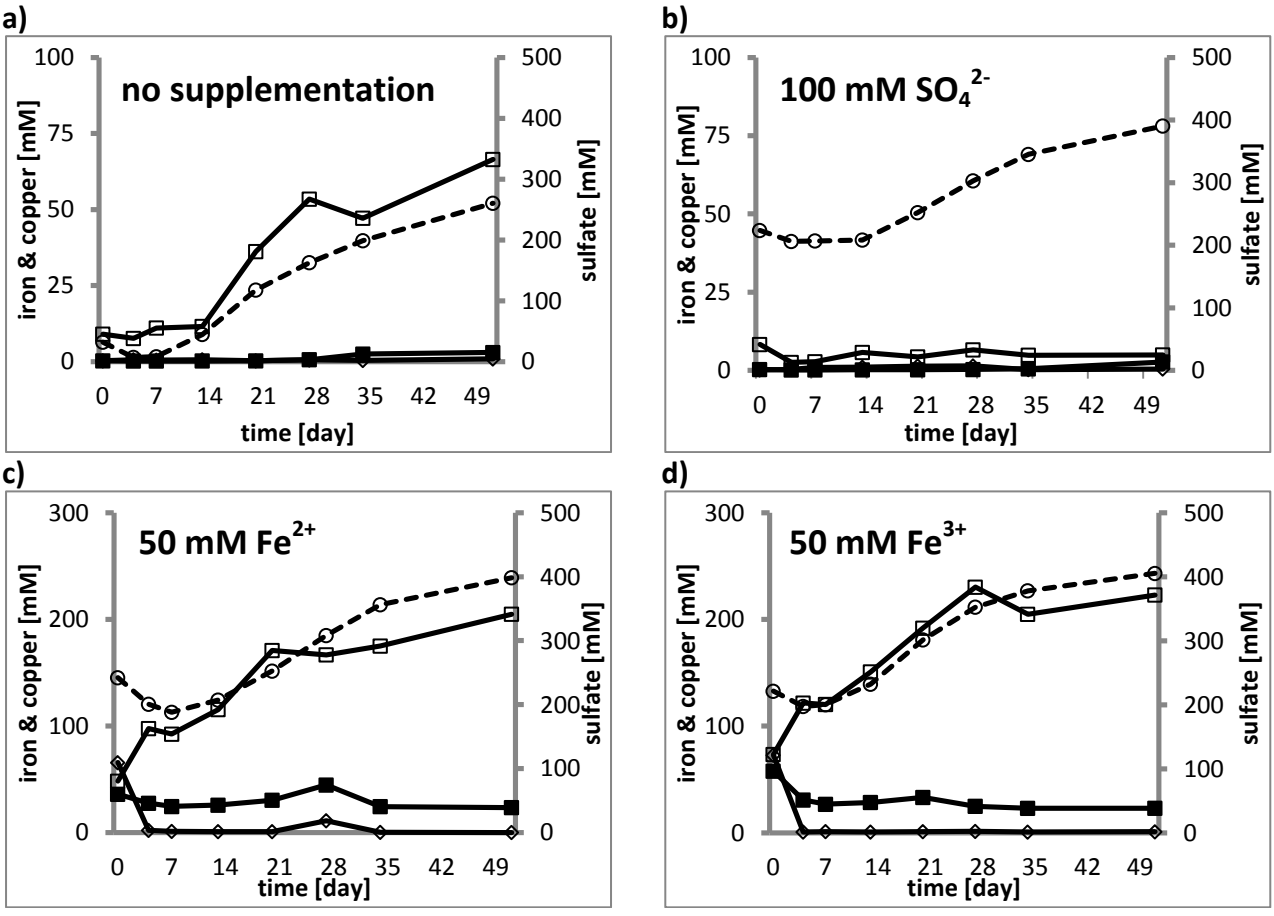

Fig. 29: Solubilized sulfate, iron and copper speciation during chemical leaching of chalcocite at 45°C supplemented with sulfate, ferrous or ferric iron. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcocite (62-200µm) were incubated at 45°C and shaken at 100 rpm; chalcocite leached a) without any supplementation, b) 100 mM sulfate (magnesium sulfate), c) 50 mM ferrous iron (iron(II) sulfate) and d) ferric iron (iron(III) sulfate) supplementation; solubilized iron (◊), copper (□), cuprous copper (■) and sulfate (○) in the leachate; in total 737 mM Cu could have been recovered.

During the leaching of chalcocite in basal salt solution 70 mM copper was solubilized, no cuprous copper or iron could be detected (Fig. 29 a). The same amount of copper was solubilized to the assays with supplementation of 100 mM sulfate. No cuprous copper or iron could be measured in these assays (Fig. 29 b). With the supplementation of ferrous or ferric iron 200 mM or 250 mM copper was solubilized, 10 % was present as cuprous copper. After 3 days no or only few iron could be detected in the leachate (Fig. 29 c and d). Immediately after the addition of ferric iron sulfate all iron was present as ferrous iron. The pH values in the assay without supplementation and with sulfate supplementation increased immediately to 4.5 and 3.5, respectively. During the experiments the pH values decreased to a value of 3. Within assays with ferrous and ferric iron supplementation the pH values increased to a value of 2.8 (Fig. 77 b, appendix).

In Fig. 30 the concentration of solubilized iron, copper and sulfate during the dissolution of covellite with a supplementation of 100 mM sulfate, 50 mM ferrous or ferric iron is shown.

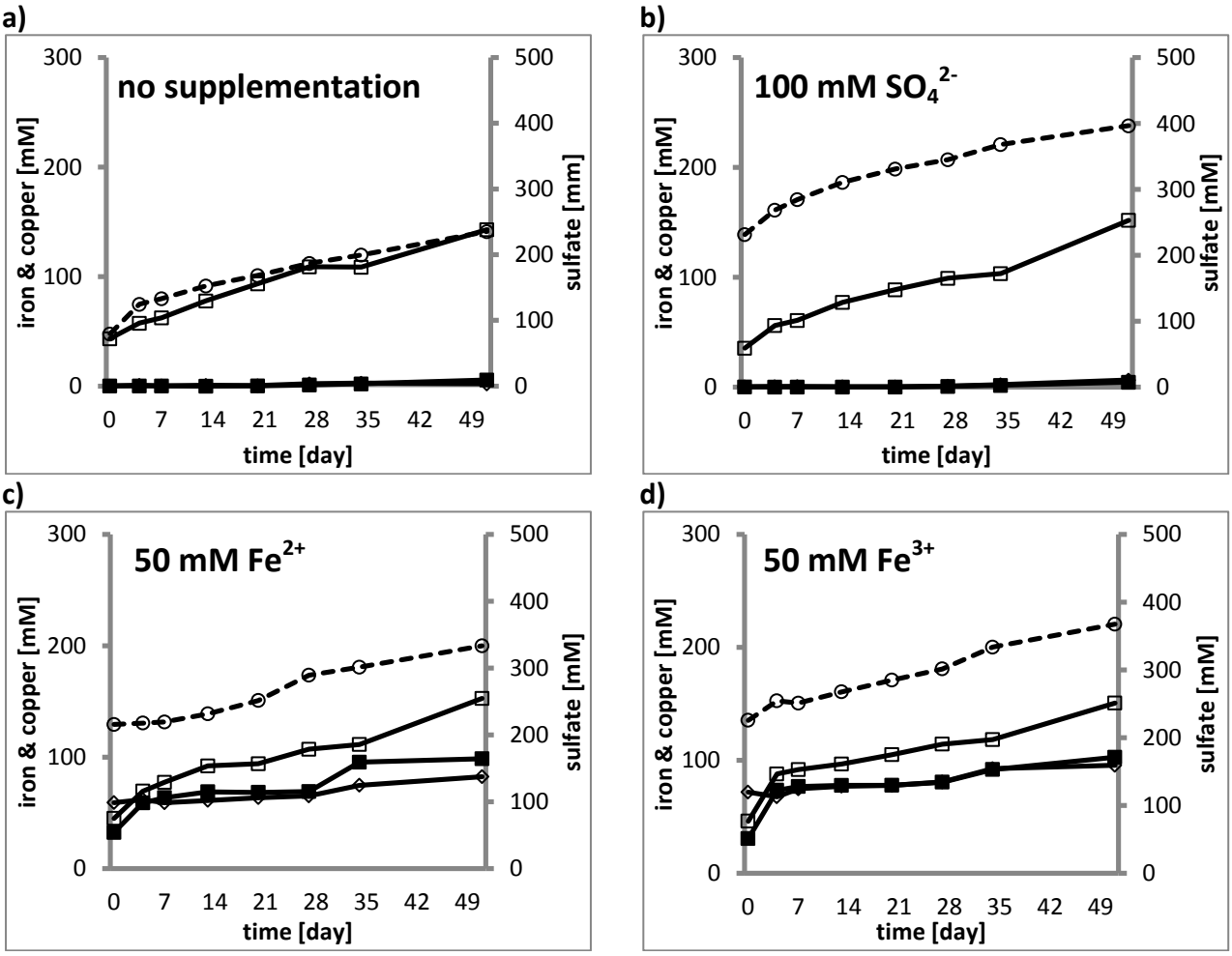

Fig. 30: Solubilized sulfate, iron and copper speciation during chemical leaching of covellite at 45°C supplemented with sulfate, ferrous or ferric iron. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % covellite (62-200µm) incubated at 45°C and shaken at 100 rpm; covellite leached a) without any supplementation, b) 100 mM sulfate (magnesium sulfate), c) 50 mM ferrous iron (iron(II) sulfate) and d) ferric iron (iron(III) sulfate) supplementation; solubilized iron (◊), copper (□) , cuprous copper (■) and sulfate (○) in the leachate; in total 802 mM Cu could have been recovered.

During the leaching of covellite in basal salt solution 150 mM copper was solubilized (Fig. 30 a). The same amount of copper was solubilized with the supplementation of 100 mM sulfate. No cuprous copper or iron could be measured in this assays (Fig. 30 b). With the supplementation of ferrous or ferric iron 250 mM copper was solubilized, 80 % was present as cuprous copper. In both assays the iron concentration was constant (Fig. 30 c and d). Immediately after the addition of ferric iron sulfate all iron was present as ferrous iron. The pH values of the assays without supplementation, with sulfate, ferrous or ferric iron supplementation increased within 3 days to values of 5, 7, 4 and 4 respectively (Fig. 77 c appendix).

Influence of cuprous copper towards bioleaching of chalcopyrite. To check whether cuprous copper has an effect on the bioleaching activity on chalcopyrite experiments with regular intervals medium exchange were conducted in order to avoid high levels of cuprous copper in the leachate.

In Fig. 31 the evolution of copper and iron species during the bioleaching of chalcopyrite (mineral load 1 %) with the moderate thermophilic enrichment culture AS is shown.

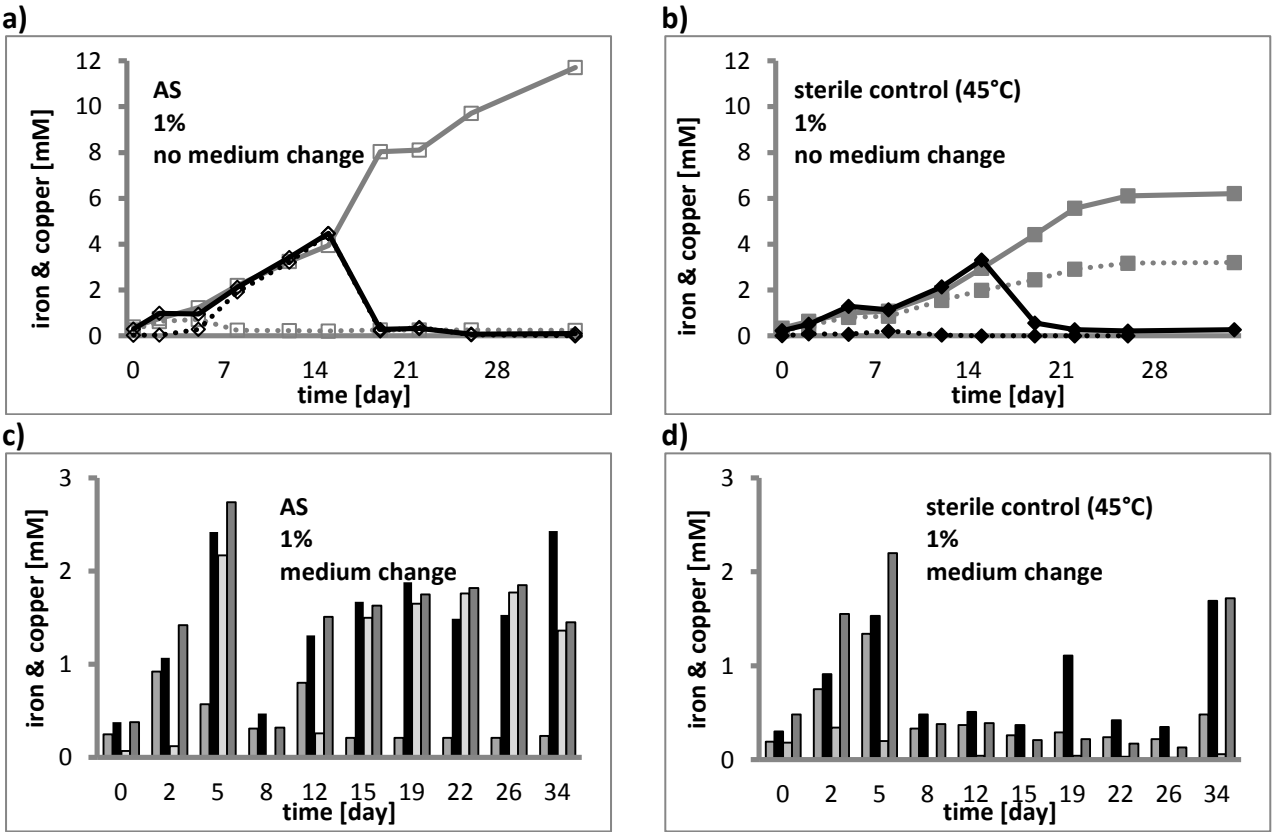

Fig. 31: Copper and iron speciation during bioleaching of chalcopyrite a) without or c) with medium change by the mesophilic enrichment culture AS. Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 1 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 45 °C and shaken at 100 rpm, basal salt solution was changed each time a sample was taken, cells were centrifuged and transferred with fresh basal salt solution to the batch back; a) total iron (black solid line ◊), ferric iron (black dashed line ◊) and total copper (gray solid line □), cuprous copper (gray dashed line □), b) sterile control without medium change, total iron (black solid line ♦), ferric iron (black dashed line ♦) and total copper (gray solid line ■), cuprous copper (gray dashed line ■); chalcopyrite leached by c) AS or d) sterile control with medium change, ferric iron (■), total iron (■) and cuprous copper (■), total copper (■); in total 52 mM Cu and 53 mM Fe could have been recovered.

During the bioleaching of chalcopyrite by the moderate thermophilic enrichment AS 12 mM copper were solubilized, low amounts of the total copper measured was present as cuprous copper. Regarding iron, 4 mM were solubilized, all of the measured iron was present as ferric iron. However, after 18 days iron could not be measured in the leachate (Fig. 31 a). In the sterile assay 6 mM copper was solubilized. During the first two weeks more than 90 % of the total copper was present as cuprous copper. After 18 days the cuprous copper concentration in the leachate remained constant (50 % of total copper), concomitantly the iron concentration was undetectable (Fig. 31 b).
During the first two weeks of chalcopyrite leaching by the moderate thermophilic enrichment culture AS with periodical medium exchange more than 70 % of the solubilized copper was present as cuprous copper, afterwards 20 % of the total copper measured in the leachate was present as cuprous copper. After the first two days 80 - 95 % of the iron measured in the leachate was present as ferric iron (Fig. 31 c). Similar amounts of iron and copper were dissolved from chalcopyrite.
In the sterile control assay 80 % of the total copper was measured as cuprous copper. Only few amounts of ferric iron could be measured in the leachate. In total 15 mM copper and iron could be solubilized due to bioleaching and 8 mM copper and iron could be solubilized by chemical leaching.

During both experimental set ups the cell counts decreased below 10^8 cells/mL. The pH during the experiment with medium exchange decreased marginal, while the pH in the experiments without medium exchange the pH of the control increased to a value of 2 after three weeks (Fig. 78, appendix).

In Fig. 32 the evolution of copper and iron species during the bioleaching of chalcopyrite (mineral load 10 %) with the moderate thermophilic enrichment culture AS is shown. During the bioleaching of chalcopyrite by the moderate thermophilic enrichment AS 25 mM copper were solubilized, few of the total copper measured was present as cuprous copper. Regarding iron, 18 mM were solubilized, after 17 days all of the measured iron was present as ferric iron. However, after three weeks no further increase in the iron concentration could be measured in the leachate (Fig. 32 a). In the sterile assay 15 mM copper was solubilized. 70 % of the total copper was present as cuprous copper. Regarding iron, 50 mM iron were solubilized, and no ferric iron was detected (Fig. 32 b).
During the first two weeks of chalcopyrite leaching by the moderate thermophilic enrichment culture AS with periodical medium exchange 70-40 % of the solubilized copper was present as cuprous copper, afterwards only few of the total copper measured in the leachate was present as cuprous copper. After the first two weeks 80 - 95 % of the iron measured in the leachate was present as ferric iron (figure 32 c). Similar amounts of iron and copper were dissolved from chalcopyrite.
In the sterile control assay 80 % of the total copper was measured as cuprous copper. Only few amounts of ferric iron could be measured in the leachate. In total 36 mM copper and 44 mM iron could be solubilized due to bioleaching and 35 mM copper and iron could be solubilized by chemical leaching.

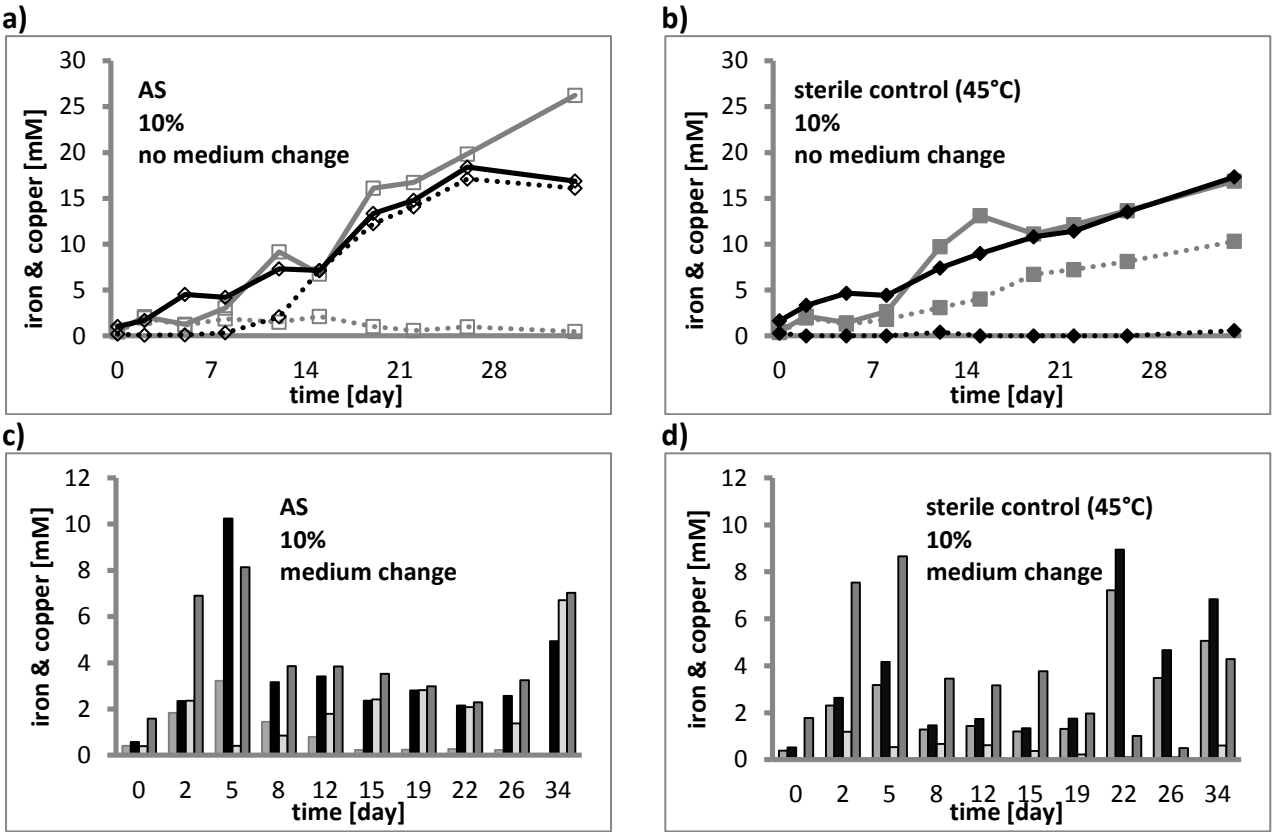

Fig. 32: Copper and iron speciation during bioleaching of chalcopyrite a) without or c) with medium change by the mesophilic enrichment culture AS. Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) were incubated at 45 °C and shaken at 100 rpm, basal salt solution was changed each time a sample was taken, cells were centrifuged and transferred with the same volume of fresh basal salt solution to the batch back; a) total iron (black solid line ◊), ferric iron (black dashed line ◊) and total copper (gray solid line □), cuprous copper (gray dashed line □), b) sterile control without medium change, total iron (black solid line ♦), ferric iron (black dashed line ♦) and total copper (gray solid line ■), cuprous copper (gray dashed line ■); chalcopyrite leached by c) AS or d) sterile control with medium change, ferric iron (■), total iron (■) and cuprous copper (■), total copper (■); in total 52 mM Cu and 53 mM Fe could have been recovered.

During both experimental set-ups the cell counts decreased below 10^8 cells/mL. However, within the assay without medium exchange after two weeks the cell density increased again. The pH during the experiment with medium exchange and without increased marginal (Fig. 78, appendix).
Fig. 33 shows CLSM-images taken during the experiment with and without medium exchange with an mineral load of 1 or 10 % of chalcopyrite. Only few cells could be imaged on chalcopyrite grains on day 8 in both assays with 10 % mineral load, after 20 days an enhanced cell attachment was visualized within assays with and without media exchange. However, after three weeks cells did not show attachment anymore in the assay without medium exchange, while few cells could be spotted on chalcopyrite within the assay with regular medium exchange. During the leaching of 1 % chalcopyrite only few cells attached to chalcopyrite could be imaged after 8 days in assays without medium exchange, while significantly more cells could be imaged at the same day in the assay with medium exchange. After 20 days in both assays similar numbers of attached cells could be visualized on chalcopyrite. However, after three weeks less cells could be imaged in the assay without medium exchange than in the one with medium exchange.

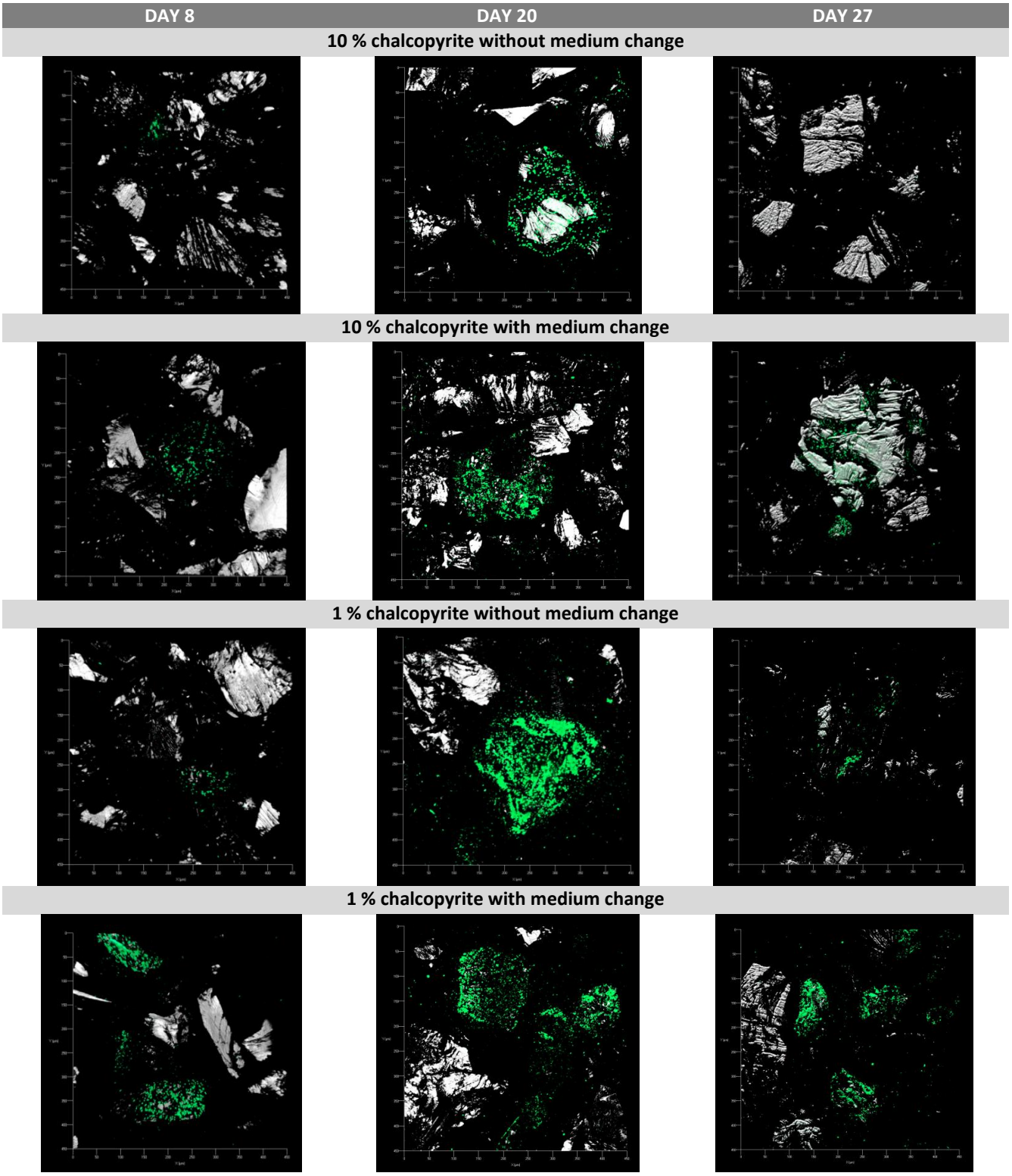

Fig. 33: Attachment of the moderate thermophilic enrichment culture AS to chalcopyrite with and without medium change visualized by CLSM. Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 1 % or 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 45 °C and shaken at 100 rpm, the basal salt solution was changed each time a sample was taken, cells were centrifuged and transferred with fresh basal salt solution to the batch back; Samples were taken after 8, 20, and 27 days and stained by Syto®9 (green).

5. Discussion

Efficiency of chalcopyrite bioleaching. Within chalcopyrite bioleaching experiments up to 24 % copper and 25 % iron could be recovered. At the beginning of this work exclusively pure cultures of *At. ferrooxidans*, *L. ferriphilum*, *Sb. thermosulfidooxidans* and *S. metallicus* were used. While these microorganisms recovered 5 % copper and iron during mesophilic leaching (28 °C), 62 % copper and 25 % iron during moderate thermophilic leaching (45 °C) and 95 % copper and 25 % iron during thermophilic leaching (65 °C) of pyrite containing copper sulfides (Fig. 4), only low copper recoveries (< 1% copper and iron during mesophilic and moderate thermophilic leaching; 2 % copper and 73 % iron during thermophilic leaching) could be gained with pure chalcopyrite (as already shown in Fig. 5).

In the literature different leaching efficiencies (expressed as copper recovery) for chalcopyrite leaching are reported. This might be due to impurities and/ or structural differences within the ore (Debernardi & Carlesi, 2013). Often pyrite is found in chalcopyrite ores. It is a well known fact that through galvanic interactions between pyrite and chalcopyrite the copper extraction is enhanced (Majuste *et al.,* 2010).

The effect of changes of the experimental leaching conditions (pH 1.5, phosphate addition) was evaluated with the objective of improving the copper recovery. During bioleaching experiments the pH tended to increase during time, due to proton consumption. At pH values of 1.9-2.2 the formation of iron precipitates is favored (Pradhan *et al.,* 2008), while pH values lower than 1.5 can prevent their formation (Debernardi & Carlesi, 2013). The addition of phosphate is enhancing the leaching efficiency (Bolan *et al.,* 1988; Zheng & Zhou, 2011). However, to avoid the precipitation of iron the pH had to be changed from 1.8 to 1.5.

In order to get higher copper recovery rates, enrichment cultures were examined. Several reports in the literature have shown that higher leaching rates are achieved using mixed rather than pure bioleaching cultures (Qui, *et al.,* 2005; Dopson *et al.,* 2007; Akcil *et al.,* 2007). During chalcopyrite leaching with the mesophilic enrichment cultures M6 and RAM 2.7 % or 3.6 % copper, respectively, could be recovered. The moderate thermophilic enrichment culture AS recovered 5.5 % copper and (after adaptation) 7.3 % copper when adapted to chalcopyrite prior to leaching experiments.

After the adaptation of the leaching set-up for mesophilic bioleaching (see Chapter 4.3) up to 9 % iron and 8 % copper could be recovered, during moderate thermophilic bioleaching (see Chapter 4.4) up to 25 % iron and 12 % copper could be leached from chalcopyrite, and during the bioleaching with thermophilic archaea (see Chapter 4.5) up to 7.4 % iron and 24 % copper could be recovered. In general, for every condition sterile controls leached only 25-50 % of the total iron and copper recovered by bioleaching.

In conventional (mesophilic) bioleaching of chalcopyrite ores often only 20 % of copper are extracted (Johnson, 2014). In this study similar recovery rates were achieved. Chalcopyrite is known as the most abundant and also as a very refractory copper ore. Compared to pyrite with a crystal lattice energy of 4260 kJ, the lattice energy of chalcopyrite is very high (17500 kJ), which explains its refractory nature (Debernardi & Carlesi, 2013). Important factors like surface attachment, shear stress and, in particular, the copper tolerance can be overcome by the adaptation of the

microorganisms to the leaching environment (Xia et al., 2008). Although the employed microorganisms had been adapted, for chalcopyrite bioleaching levels of up to 30 mM copper have been reached. Experiments conducted with the employed microorganisms to examine the copper tolerance showed cell growth (with iron sulfate or sulfur, data not shown) up to a copper concentration of 20 mM.

Despite the fact that bioleaching microorganisms could be helpful for chalcopyrite dissolution, many experiments have been conducted under controlled redox potential conditions (Sandström *et al.,* 2005; Hiroyoshi *et al.,* 2007 and 2008; Ahmadi *et al.,* 2011; Khoshkhoo *et al.,* 2014; Rodrígiez *et al,.* 2015). Third *et al.* (2000) concluded that the redox potential is more effective than the activity of bacteria. High ferric iron concentrations (or a high redox potential) lead to poor leaching results. If the produced ferric iron quantity is higher than the consumed one, bacteria can be detrimental for chalcopyrite dissolution. However, below potentials of 600-700 mV SHE chalcopyrite is always activated, while at higher redox potentials the surface of chalcopyrite is passivated (Viramontes-Gamboa *et al.,* 2010). In the experiments done in this study redox potential was not controlled, but during the leaching of chalcopyrite redox potential values above 600 mV SHE have been measured most likely explaining the relatively low recovery rates.

Concerning chalcopyrite (bio)leaching the phenomenon of passivation has been intensively discussed in literature (Hackl *et al.* 1995; Debernardi & Carlesi, 2014). Sometimes only 10 - 25 % of the copper in chalcopyrite are released, before the reaction slows down or stops due to passivation (Keeling *et al.,* 2006). There is no general consent about the nature of the passivation, but it is accepted that the formation of one or more inhibiting layers limits the reactivity of the surface and decreases copper extraction rates (Watling, 2006; Debernardi & Carlesi, 2014). As components of a passivation layer elemental sulfur (Munoz et al., 1979; Dutrizac, 1989; Klauber *et al.,* 2001), metal-deficient sulfides (Biegler & Swift, 1979; Holliday & Richmand, 1990), polysulfides (Hackl *et al.,* 1996), and iron precipitates (Stott *et al.,* 2000; Sandström *et al.,* 2005; Córdoba *et al.,* 2008) are reported. Sulfur is rather forming a porous layer of the surface on chalcopyrite and can also be oxidized to sulfate by acid producing sulfur oxidizing bacteria (Vargas *et al.,* 2014). Chalcopyrite is dissolved by the attack of ferric ions and protons, since it is an acid soluble mineral (Vera *et al.,* 2013). During the bioleaching experiments in this study the pH tended to increase during time due to proton consumption. The pH increase (up to 2.5) and the non-stoichiometric proportions of copper and iron might be an indication for iron precipitation during the experiments. Although it is known that iron and copper do not dissolve in a ratio of 1:1 , but rather 2:1 (Linge, 1976), 4:1, or 5:1 (Biegler & Swift, 1979; Holliday & Richmand, 1990), in the experiments performed in this thesis more copper than iron was released. Furthermore, during the experiments with a mineral load of 1% after two weeks iron could not be detected anymore in solution and sulfate levels did not increase during leaching. Several metal-deficient sulfides may arise during chalcopyrite dissolution such as $Cu_{1-x}Fe_{1-y}S_{2-z}$ (y>x) and Cu_XS (0.7< x < 1) (Warren *et al.*, 1982; Biegler and Horne, 1985; Holliday & Richmond, 1990; Hackl *et al.,* 1995).

Along with the hydrolysis of iron at pH 2 - 2.5, precipitations like goethite [FeOOH] and, in the presence of sulfate, also schwertmannite [$Fe_8O_8(OH)_6(SO_4)$] occur. Jarosite [$MFe_3(SO_4)_2(OH)_6$] formation can be triggered by the presence of goethite and/ or iron hydroxides [$Fe(OH)_X$] and

cations (K^+, Na^+ and/ or NH_4^+), (Klauber *et al.,* 2001). During the experiments red-brown precipitates could be observed; X-ray diffraction (XRD) spectroscopy proved the presence of jarosite, although not quantitatively (data not shown).

Calorimetric measurements on chalcopyrite degradation. Microcalorimetry had been proven to be useful for measuring the bioleaching activity in pyrite oxidizing cultures (Schröter & Sand, 1989; Schippers *et al.,* 1998; Rohwerder *et al.,* 1998). Rohwerder *et al.* 1998 plotted the microcalorimetrically measured and (on the basis of dissolved iron) calculated reaction energies ($\Delta_r U$) in one graph. Both values were close to each other (less than 20% deviation). In this study (Table 9), the measured heat output ($^{\mu W}/_{g}$) was converted to thermal energy ($^{J}/_{g*d}$) and the detected copper concentration in the leachate was expressed as oxidation rate ($^{mM}/_{g*d}$). Then the thermal energy was divided by the oxidation rate resulting in the reaction energy (calculated) that was released by the amount of copper measured in the leachate. This calculated reaction energy ($\Delta_r U$) was compared to the standard reaction energy ($\Delta_r H^f$) of chalcopyrite dissolution given in the literature and the ratio was given.
However, during the degradation of pyrite for each mol pyrite one mol iron is released (Schröter & Sand, 1989), but for each mol chalcopyrite one mol iron as well as one mole copper are released (Dixon, 2000). In this thesis, for the thermodynamic calculations copper ions seemed to be more reliable to estimate chalcopyrite dissolution rather than iron. Experiments shown in table 13 demonstrated that referring to copper, the amount of leached ions (calculated on the basis of weight loss determination of the substratum) matches the concentration of measured ions in the leachate during bioleaching (10% mineral load at 28 °C or 45 °C) in a significantly better way than in case of iron.
Up to 82 %, 90 % or 85 % of the standard reaction energy of chalcopyrite dissolution were detected via microcalorimetry for mesophiles, moderate thermophiles or thermophiles, respectively. If the calculation of the reaction energy leads to values lower than estimated, not all bonds within the chalcopyrite crystal seem to be broken and not all of the energy was released. On the other hand, the occurrence of precipitations (especially caused by iron and copper ions) may lead to an overestimation of the reaction energy.
In this thesis different chalcopyrite ores were employed and all of them showed similar leaching characteristics and heat output values. This shows that microcalorimetriy can be applied on different chalcopyrite ores.
Summing up microcalorimetry is a suitable technique for the measurement of chalcopyrite leaching by microorganisms growing in different temperature ranges.

Visualization of leaching biofilms on chalcopyrite. The biofilm formation on chalcopyrite was followed by CLSM. The images of microbially colonized chalcopyrite showed that the population density differed with the conditions under which the microorganisms were pre-grown. Iron sulfate and pyrite pre-cultivated microorganisms showed higher attachment to chalcopyrite than cultures already adapted to chalcopyrite.

Sulfur pre-grown cells were easily detachable from the mineral during the staining procedure. This may be due to some differences within the EPS. Sulfur grown *At. ferrooxidans* cells exhibit higher hydrophobic surface properties than iron or pyrite grown cells and do not attach to pyrite particles (Gehrke *et al.,* 1998). A high coverage of chalcopyrite by attached microorganisms seems not fully correlating with the copper extraction rate . Contrary to this, during pyrite bioleaching an enhanced biofilm formation positively correlates with its dissolution (Sand *et al.,* 1995; Florian *et al.,* 2011).

(Bio)leaching of Chalcocite and Covellite. Chalcocite is an acid-soluble mineral which can be leached by ferric iron as well as protons (Brierley & Kuhn, 2010). Chalcocite leaching showed in the mesophilic temperature range up to 17 % copper recovery by chemical leaching and bioleaching. In contrast, with moderate thermophilic leaching only 17 % copper were leached chemically, but up to 41 % was due to bioleaching. The used ore seemed to be considerabley acid consuming, since during the experiments the pH increased to values above 4. Even daily titration to a pH below 2 was not successful to keep the pH value stable. Copper concentrations of up to 150 mM were reached during chalcocite bioleaching. The increased pH in association with the high copper concentration made cell growth impossible. Missing cell growth, but also the deceleration of chalcocite dissolution through the formation of the almost inert “blue-remaining” covellite (Leahy *et al.,* 2007) could be the reasons for the relatively poor copper extraction. In contrast, it has been reported that chalcocite is dissolved stepwise. 40 % of the contained copper are dissolved during the first stage which proceeds quickly and 60 % are released in the second stage which is considerable slower than the first one (Brierley & Kuhn, 2010). Extraction rates of 50 - 90 % were achieved during chalcocite leaching.

Copper recovery during bioleaching of covellite (Chapter 4.6) was similar for the mesophilic and moderate thermophilic temperature ranges, i.e. 2 - 15 %. Copper recoveries of covellite range from 20 % for mesophiles up to 80 % for thermophiles (Acar *et al.,* 2005). During the experiments the pH increased slightly and copper concentrations of 100 mM were reached in the leachate. Due to the small quantities of iron (3%) and the increasing pH an oxidant was missing. However, cuprous ions were not detectable. Covellite is known to be a refractory copper sulfide especially in the mesophilic temperature range (Miki *et al.,* 2011). During the leaching of covellite several copper sulfide intermediates are formed, like “blue-remaining” covellite (Acar *et al.,* 2005). In general secondary covellite is leached easier than the primary one. Addition of pyrite can improve the dissolution significantly (Cooper & Dixon, 2006).

For mesophilic chalcocite leaching 5 - 12 % and for moderate thermophilic leaching 32 – 38 % of the standard reaction energy could be detected via microcalorimetry. For covellite leaching 10 – 15 % of the standard reaction energy could be detected regardless of the temperature. By calorimetry only the net heat output signal can be measured. During this study several problems concerning chalcocite and covellite leaching arose . The (high) pH values were not ideal for leaching, but favorable for iron precipitation. Extremely high amounts of copper were dissolved in the beginning of the experiments leading to a decrease in cell counts and less activity. This might have had an

impact on the caloric data, meaning that the thermal output cannot be reduced exclusively by the dissolution of the mineral.
Though microcalorimetry is suitable for the assessment of bioleaching activity with chalcopyrite ores, in this study we conclude that it is rather not a suitable technique for chalcocite or covellite bioleaching. However, this might be related to the minerals used in this work and some other sources of chalcocite and covellite need to be studied in the future.

Influence of cuprous copper on copper sulfide leaching. During chalcopyrite leaching experiments considerable amounts of cuprous copper were determined within the leachates. Cuprous copper is known to be more toxic than cupric copper (Beswick *et al.,* 1976; Holland & White, 1988).
Cuprous ions are known to be unstable in aquatic environments (Matoha *et al.,* 2005), unless there are complexed by chloride (Parker *et al.,* 1981) or by solvents like CH_3CN (Georgopoulos *et al.,* 2001) or acetonitrile (Altermatt *et al.,* 1968). However, cuprous copper has been found in seawater (Moffettt *et al.,* 1988; Millereo *et al.,* 1991), urban river water (Glazewski *et al.,* 1996), fog water (Xue *et al.,* 1991) and rain water (Kieber *et al.,* 2004). The uptake of copper by rainbow trout gills had been reported due to a reduction of cupric to cuprous copper prior to membrane transport (Bopp *et al.,* 2008), it was found that this reaction was catalyzed by sulfhydryl groups (Felsenfeld, 1960; Bogdanova *et al.,* 1991).
The presence of cuprous ions in leaching environments has been reported previously by Muir *et al.,* 1975 (cupric chloride leaching) and Mc Donald *et al,.* 1984 (electrorefining involving organic nitriles) and recently by Anwar *et al.,* 2000 (sulfate-based leaching). The reason for the existence of cuprous copper in sulfate-based solutions could be related to iron species (Matocha *et al.,* 2005). A reduction of cupric ions to cuprous ions is possible by ferrous iron (Wegwe, 1999). In contrast, ferric ions are known to oxidize cuprous ions (Debernardi *et al.,* 2012).
Based on the bichinonic acid method for colorimetric determination of soluble copper speciations significant amounts (up to 80 % of the total concentration) of copper were found to be present as cuprous copper. The more ferrous iron was present, the more cuprous copper was found. During later stages of leaching, when almost all iron was present as ferric iron, only minor amounts of cuprous copper could be measured. It has to be critically noted that the applied colorimetric method used in this study uses a precipitation step for ferric iron, shifting the chemical equilibrium towards ferrous iron (Debernardi *et al.,* 2012). However, the stability of cuprous copper in leaching environments is mostly driven by the ferrous iron concentration. Further research has to be done to investigate the influence of ferrous/ ferric iron on the stability of cuprous copper measured by the bichinchonic acid method. This might be decisive for the collection of reliable caloric data. Further test needs to be done to determine in how far the precipitation step is influencing the Cu^{1+}/Cu^{2+} ratio and/ or is shifting the balance towards cuprous copper.

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APPENDIX

Element analysis of mineral sulfides

Tab.. 20: Elemental analysis by x-ray fluorescence spectroscopy, of Chalcopyrite, chalcocite and covellite, marked elements (*) can inhibit the bioleaching process.

Element	Unit	Chalcopyrite	Pyrite	Covellite	Element	Unit	Chalcopyrite	Pyrite	Covellite
Na_2O	%	< 0,26	< 0,21	0,113	**Zr**	µg/g	1,3	4,9	0,9
MgO	%	0,664	1,029	0,922	**Nb**	µg/g	1,7	1,7	0,8
Al_2O_3	%	< 0,026	< 0,0069	2,515	**Mo***	µg/g	< 0,8	< 0,6	< 0,2
SiO	%	1,751	18,84	14,58	**Ag***	µg/g	90,5	930,6	5,8
P_2O_5	%	< 0,014	< 0,013	< 0,013	**Cd**	µg/g	31,7	11,6	0,8
S	**%**	**16,86**	**13,06**	**11,73**	**In**	µg/g	3,2	< 0,2	< 0,1
Cl	%		< 0,00001	< 0,00001	**Sn**	µg/g	154,0	< 0,6	4,6
K_2O	%	< 0,012	< 0,7361	0,01086	**Sb**	µg/g	13,8	50,9	6,8
CaO	%	0,02700	1,136	0,03678	**Te***	µg/g	< 1,3	9,2	10,8
TiO_2	%	< 0,0083	0,03736	0,05709	**I**	µg/g		< 1,5	< 1,5
V	µg/g	< 15	21,9	106	**Cs**	µg/g	< 1,3	< 1,3	3
Cr	µg/g	73,9	165,7	65,7	**Ba**	µg/g	< 1,7	59,1	761,8
MnO	%	< 0,00005	0,2902	< 0,00009	**La**	µg/g	2,5	< 2,4	1,3
Fe_2O_3	**%**	**29,62**	**26,54**	**1,269**	**Ce**	µg/g		8,8	< 1,8
Co	µg/g	< 130	< 78	< 160	**Pr**	µg/g		< 6,2	12
Ni	µg/g	197	< 9,6	39,6	**Nd**	µg/g		< 4,2	11,5
Cu	**µg/g**	**179500**	**86860**	**143200**	**Sm**	µg/g		15,9	< 2,2
Zn	µg/g	254,9	319,7	94,3	**Yb**	µg/g		99,6	< 11
Ga	µg/g	18,8	2,2	6,7	**Hf**	µg/g	< 0,1	98	< 0,1
Ge	µg/g	2,0	2,8	20,9	**Ta**	µg/g	< 1,5	< 1,5	< 1,5
As*	µg/g	147,4	463,9	299,6	**W**	µg/g		318	< 17
Se*	µg/g	4,2	6,6	1,6	**Hg**	µg/g	< 3,1	4,4	< 2,2
Br	µg/g		< 1,9	< 1,0	**Tl**	µg/g	2,2	5,2	< 0,8
Rb	µg/g	1,8	17,1	< 0,2	**Pb**	µg/g	646,5	240,7	11,8
Sr	µg/g	3,0	4,2	20,4	**Bi**	µg/g	< 3,6	52,4	< 1,3
Y	µg/g	2,4	1,2	0,5	**Th**	µg/g	15,9	3,7	< 0,6
Sr	µg/g	3,0			**U**	µg/g	< 2,1	< 1,7	< 0,5

Screening of appropriate microorganisms

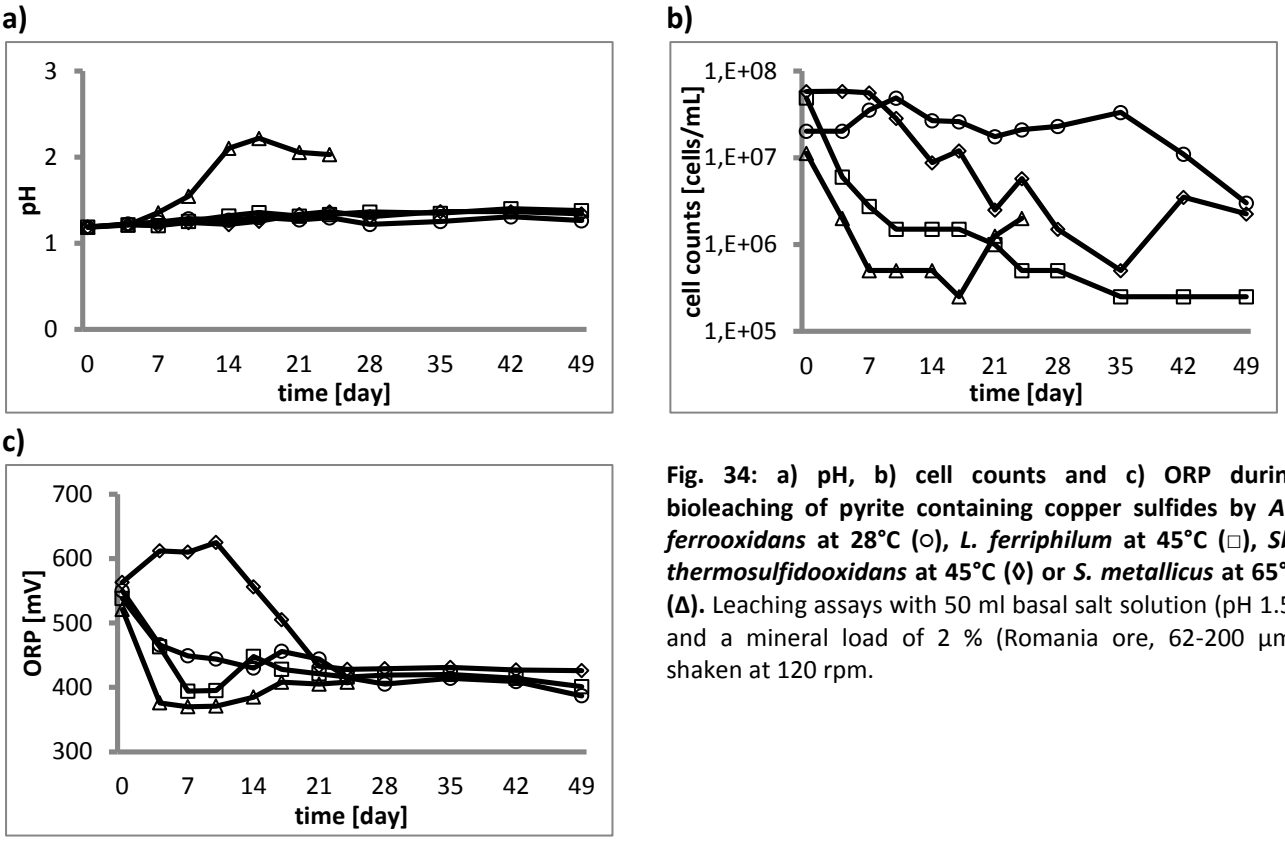

Fig. 34: a) pH, b) cell counts and c) ORP during bioleaching of pyrite containing copper sulfides by *At. ferrooxidans* at 28°C (○), *L. ferriphilum* at 45°C (□), *Sb. thermosulfidooxidans* at 45°C (◊) or *S. metallicus* at 65°C (Δ). Leaching assays with 50 ml basal salt solution (pH 1.5) and a mineral load of 2 % (Romania ore, 62-200 µm) shaken at 120 rpm.

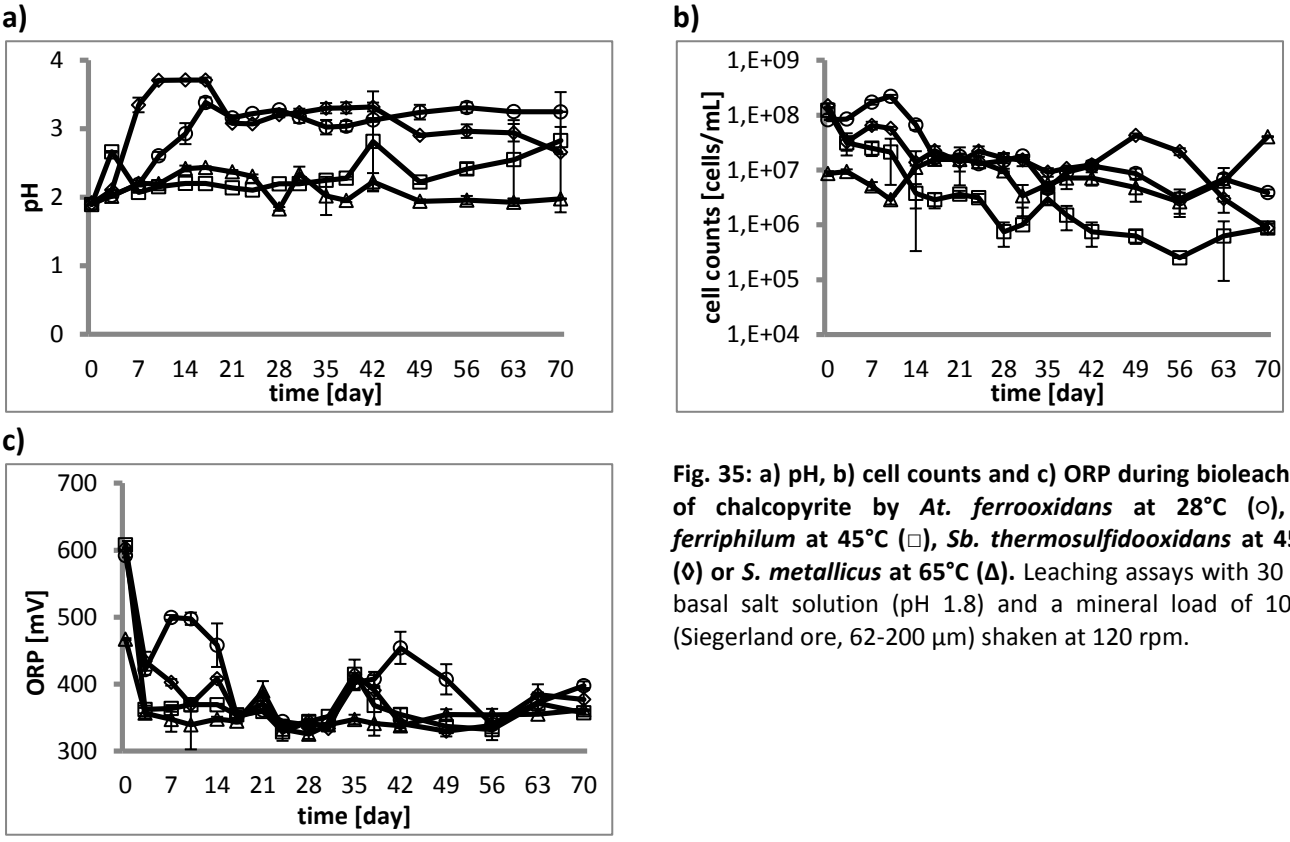

Fig. 35: a) pH, b) cell counts and c) ORP during bioleaching of chalcopyrite by *At. ferrooxidans* at 28°C (○), *L. ferriphilum* at 45°C (□), *Sb. thermosulfidooxidans* at 45°C (◊) or *S. metallicus* at 65°C (Δ). Leaching assays with 30 mL basal salt solution (pH 1.8) and a mineral load of 10 % (Siegerland ore, 62-200 µm) shaken at 120 rpm.

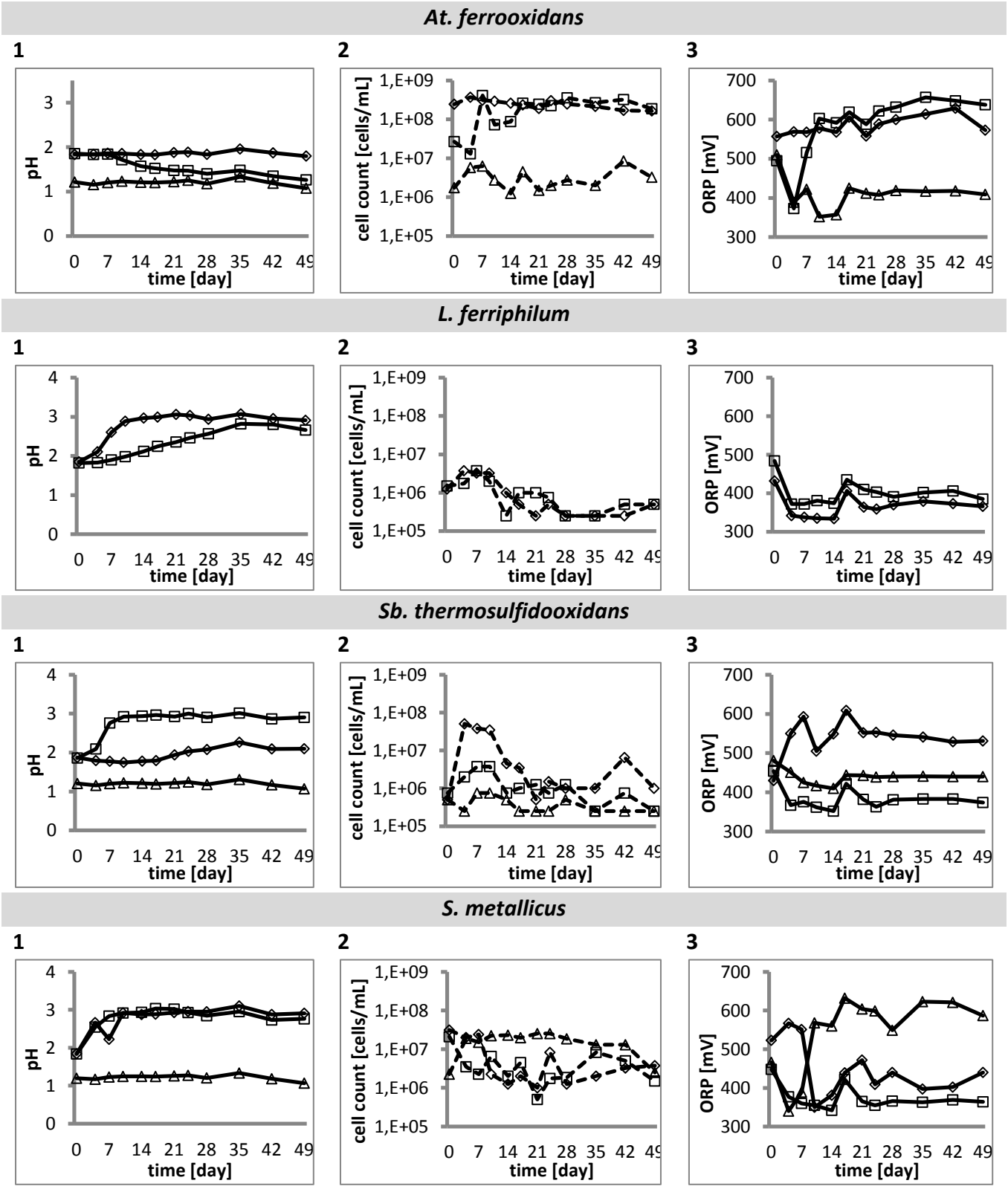

Fig. 36: pH (1), cell counts (2) and ORP (3) during chalcopyrite bioleaching with *At. ferrooxidans* at 28°C , *L. ferriphilum* at 45°C, *Sb. thermosulfidooxidans* at 45°C or *S. metallicus* at 65°C in basal salt solution with a pH of 1.8 without (◊) and with (□) additional phosphate supplementation (1 mM phosphate) or a pH of 1.5 (Δ). Leaching assays with 100 mL (pH 1.8 with and without additional phosphate) or 500 mL (pH 1.5) basal salt solution each shaken at 120 rpm. Assays without phosphate supplement were loaded with 2 % mineral (Harz, 62-200 µm) and assays with phosphate supplement were loaded with 10% mineral.

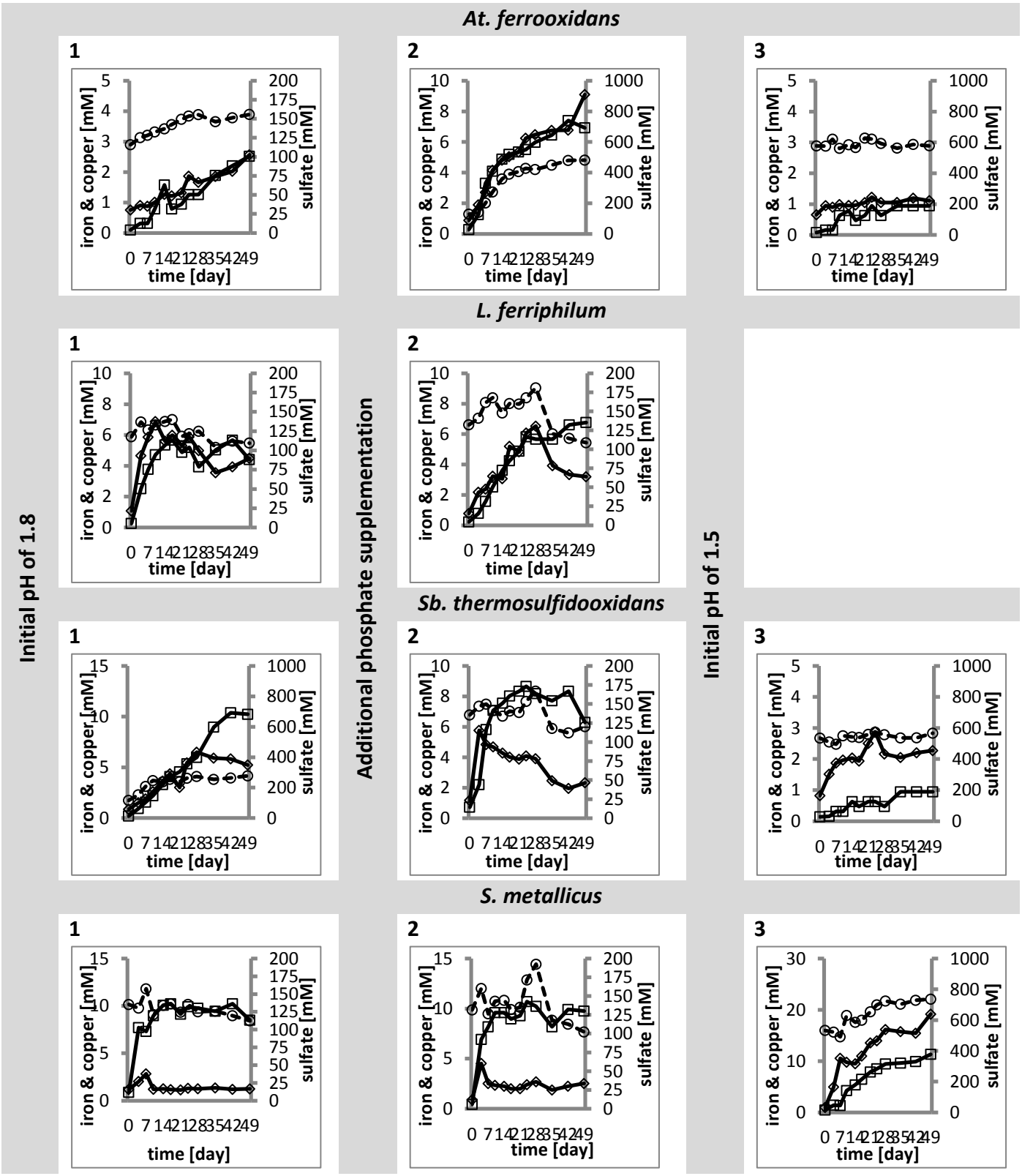

Fig. 37: Solubilized copper (□), iron (◊) or sulfate ions (○) during chalcopyrite bioleaching with *At. ferrooxidans* at 28°C, *L. ferriphilum* at 45°C, *Sb. thermosulfidooxidans* at 45°C or *S. metallicus* at 65°C in basal salt solution with a pH of 1.8 without (1) or with (2) additional phosphate supplementation (1 mM phosphate) or a pH of 1.5 (3). Leaching assays with 100 mL (pH 1.8 with and without additional phosphate) or 500 mL (pH 1.5) basal salt solution each shaken at 120 rpm. Assays without phosphate supplement were loaded with 2 % mineral (Harz, 62-200 µm) and assays with phosphate supplement were loaded with 10% mineral.

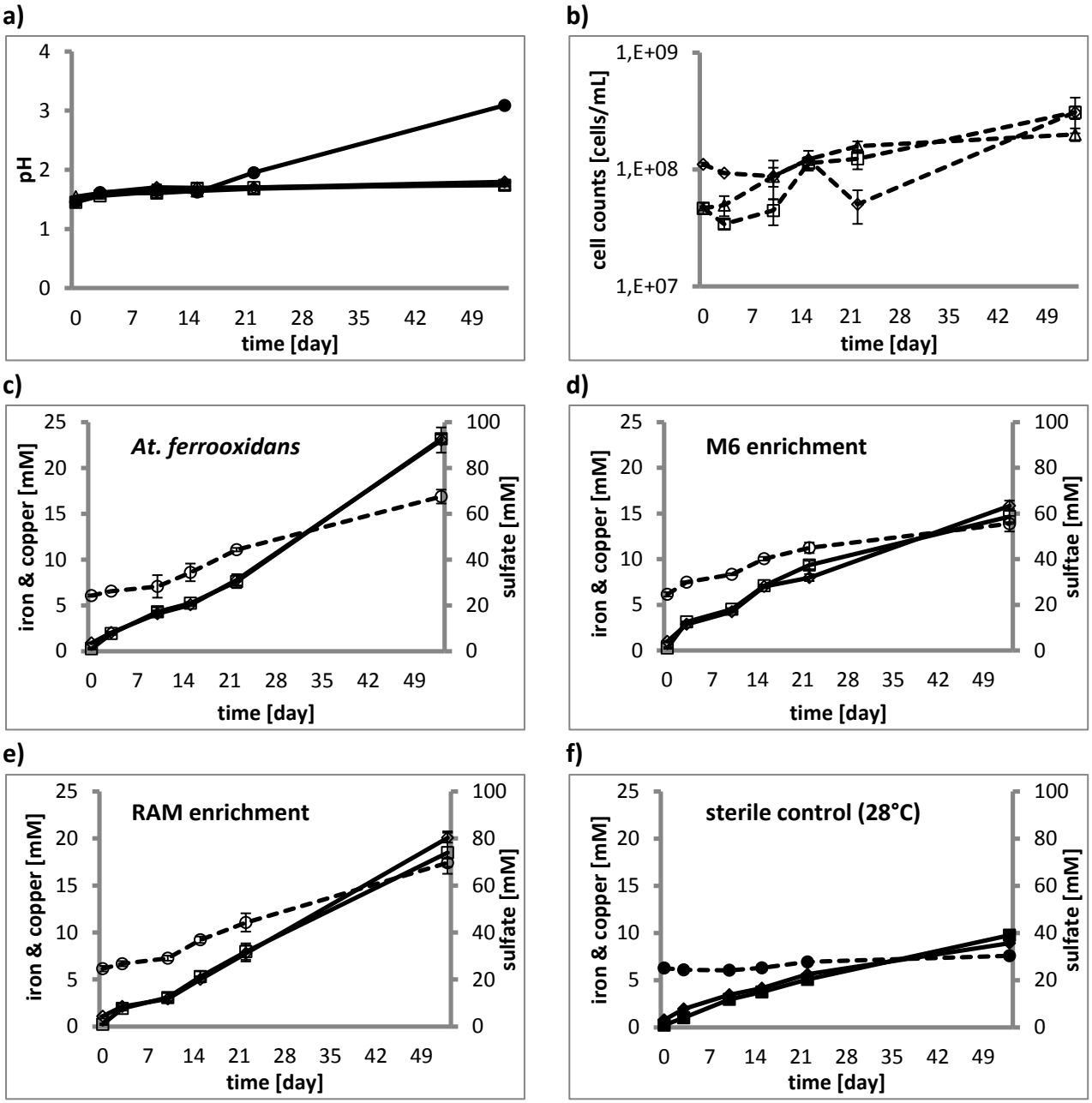

Fig. 38: Chemical leaching and bioleaching of chalcopyrite at 28°C by *At. ferrooxidans*, mesophilic enrichment cultures M6 or RAM. Leaching assays with 30 ml basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) shaken at 120 rpm; **a)** pH and **b)** cell counts of *At. ferrooxidans* (◊), M6 (□), RAM (Δ) or the chemical control (●); solubilized iron (◊), copper (□) or sulfate (○) in the leachate of **c)** *At. ferrooxidans* **d)** M6 or **e)** RAM**; f)** dissolved iron (♦), copper (■) or sulfate (●) in the leachate of the chemical control assay.

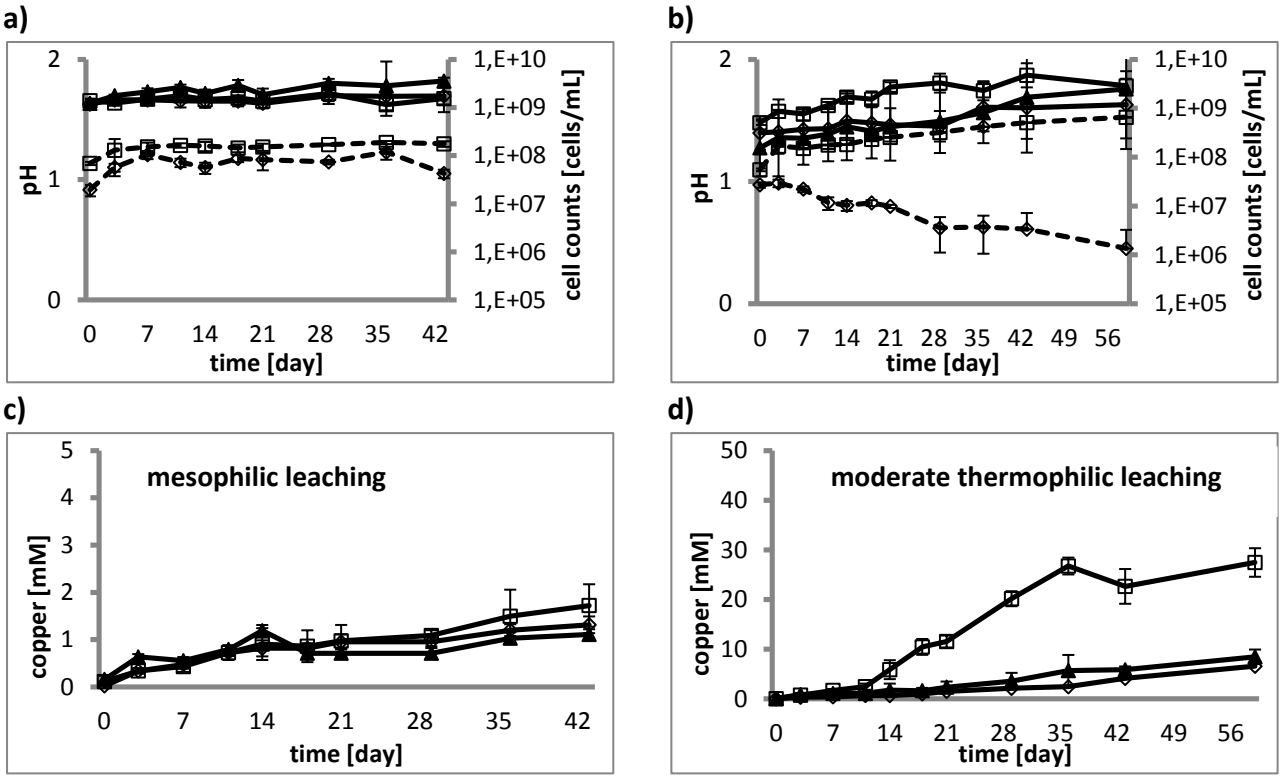

Fig. 39: Chemical leaching and bioleaching of chalcopyrite at 28°C or 45°C by *At. ferrooxidans*, the mesophilic enrichment culture M6, *Sb. thermosulfidooxidans* or the moderate thermophilic enrichment culture AS. Leaching assays with 30 ml basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 28 °C (*At. ferrooxidans* or M6) or at 45°C (*Sb. thermosulfidooxidans* or AS) shaken at 120 rpm; **a)** pH and cell counts in the mesophilic assays with *At. ferrooxidans* (◊), M6 (□) or the chemical control (▲); **b)** pH and cell counts in the moderate thermophilic assays with *Sb. thermosulfidooxidans* (◊), AS (□) or the chemical control (▲); **c)** solubilized copper in the mesophilic assays with *At. ferrooxidans* (◊), M6 (□) or the chemical control (▲); **d)** solubilized copper in the moderate thermophilic assays with of *Sb. thermosulfidooxidans* (◊), AS (□) or the chemical control.

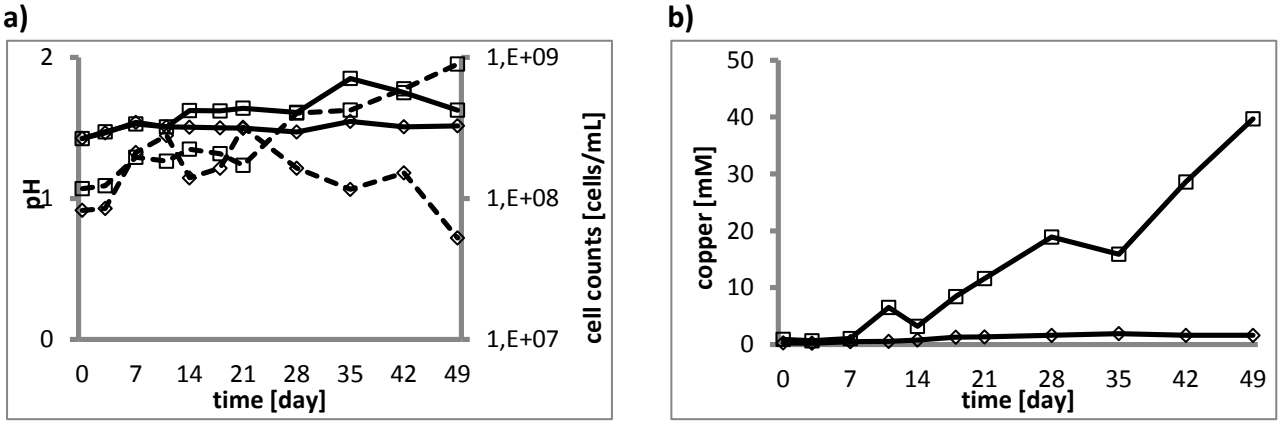

Fig. 40: Chemical leaching and bioleaching of chalcopyrite at 28°C or 45°C by *At. ferrooxidans* or the moderate thermophilic enrichment culture AS. Leaching assays with 30 ml basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 28 °C (*At. ferrooxidans*) or at 45°C (AS) shaken at 120 rpm; **a)** pH and cell counts of the leaching assays with *At. ferrooxidans* (◊) or AS (□); **b)** solubilized copper in the leaching assays with *At. ferrooxidans* (◊) or AS (□).

Degradability of chalcopyrite ores

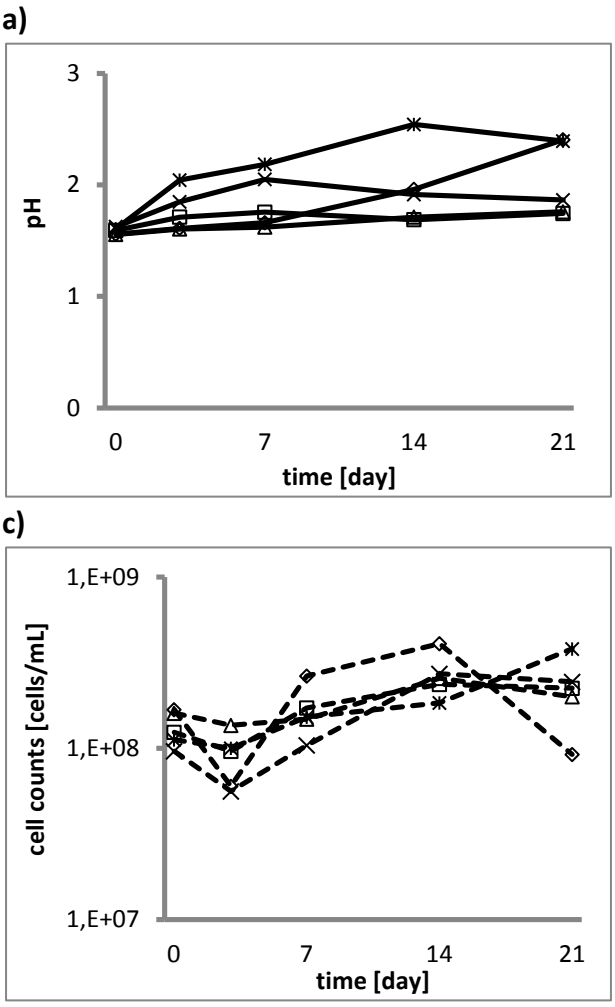

Fig. 41: pH and cell counts during chemical leaching or bioleaching of chalcopyrite ores originated from different sampling sites at 45°C by the moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (62-200 µm) shaken at 120 rpm; **a)** pH of AS assays with ores from Siegerland (◊), Peru (□), Sweden (Δ), Harz (x) or Romenia (ж); **b)** pH of chemical control assays with ores from Siegerland (♦), Peru (■), Sweden (▲), Harz (x) or Romania (ж); **c)** cell counts of AS assays with ores from Siegerland (◊), Peru (□), Sweden (Δ), Harz (x) or Romania (ж);

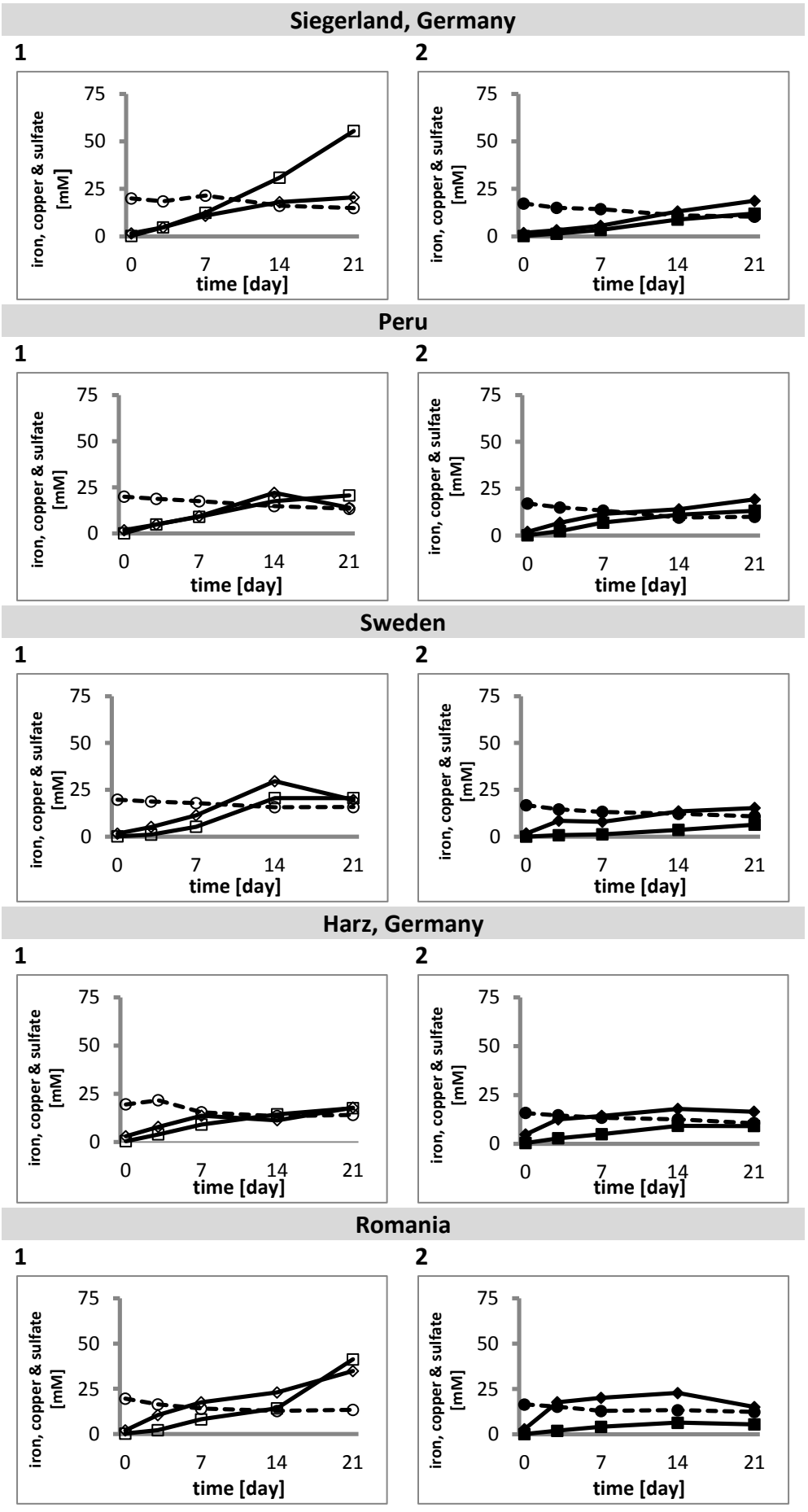

Fig. 42: Solubilized iron, copper or sulfate during chemical leaching and bioleaching of chalcopyrite ores originated from different sampling sites at 45°C by the moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (62-200 µm) shaken at 120 rpm; **1)** iron (◊), copper (□) and sulfate (○) in leaching assays with AS **2)** iron (♦), copper (■) or sulfate (●) in chemical control assays.

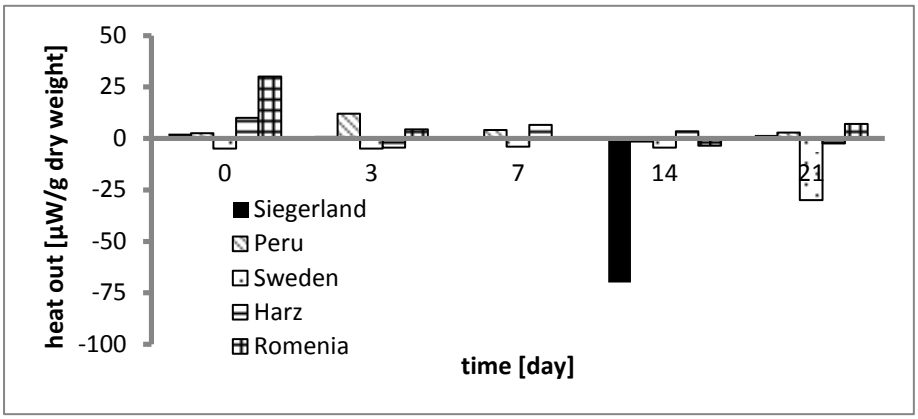

Fig. 43: Microcalorimetric determination of the heat output during chemical leaching of chalcopyrite ores originated from different sampling sites at 45°C. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (62-200 µm) shaken at 120 rpm.

Calorimetric measurements of chalcopyrite degradation at 28 °C

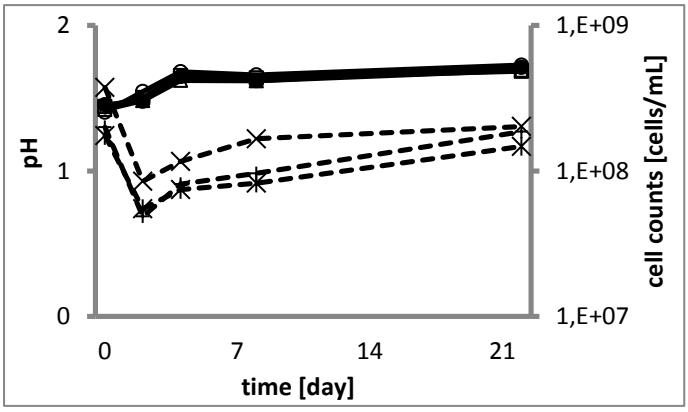

Fig. 44: pH and cell counts during chemical leaching or bioleaching of chalcopyrite at 28°C by *At. ferrooxidans* or the mesophilic enrichment cultures M6 or RAM. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (62-200 µm) shaken at 100 rpm; pH of *At. ferrooxidans* assay (○), M6 assay (□), RAM assay (Δ) or chemical control assay (●), cell counts of *At. ferrooxidans* assay (x), M6 assay (ж) or RAM assay (+).

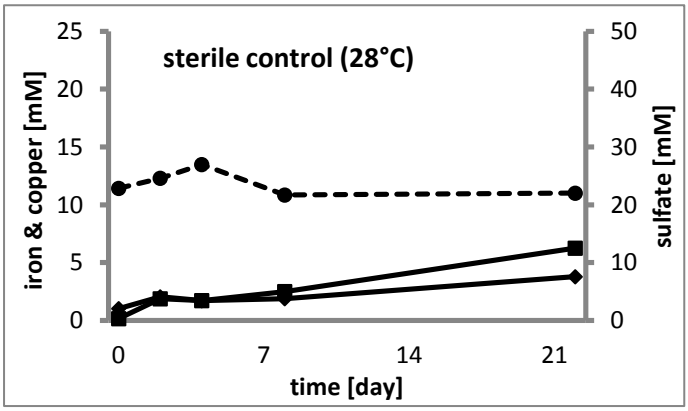

Fig. 45: Chemical leaching of chalcopyrite at 28°C. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (Siegerland ore, 62-200 µm) shaken at 100 rpm; solubilized iron (♦), copper (■) and sulfate (●) in the leachate.

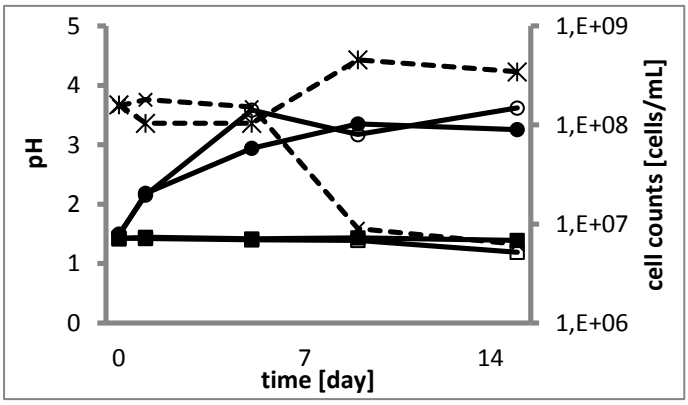

Fig. 46: pH and cell counts during chemical leaching or bioleaching of chalcopyrite or pyrite at 28°C by the mesophilic enrichment culture RAM. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (Siegerland ore, Romania ore, 62-200 µm) shaken at 100 rpm; pH of inoculated chalcopyrite assay (○) or pyrite assay (□) or chemical control assay with chalcopyrite (●) or pyrite (■), cell counts of chalcopyrite assay (x) or pyrite assay (ж).

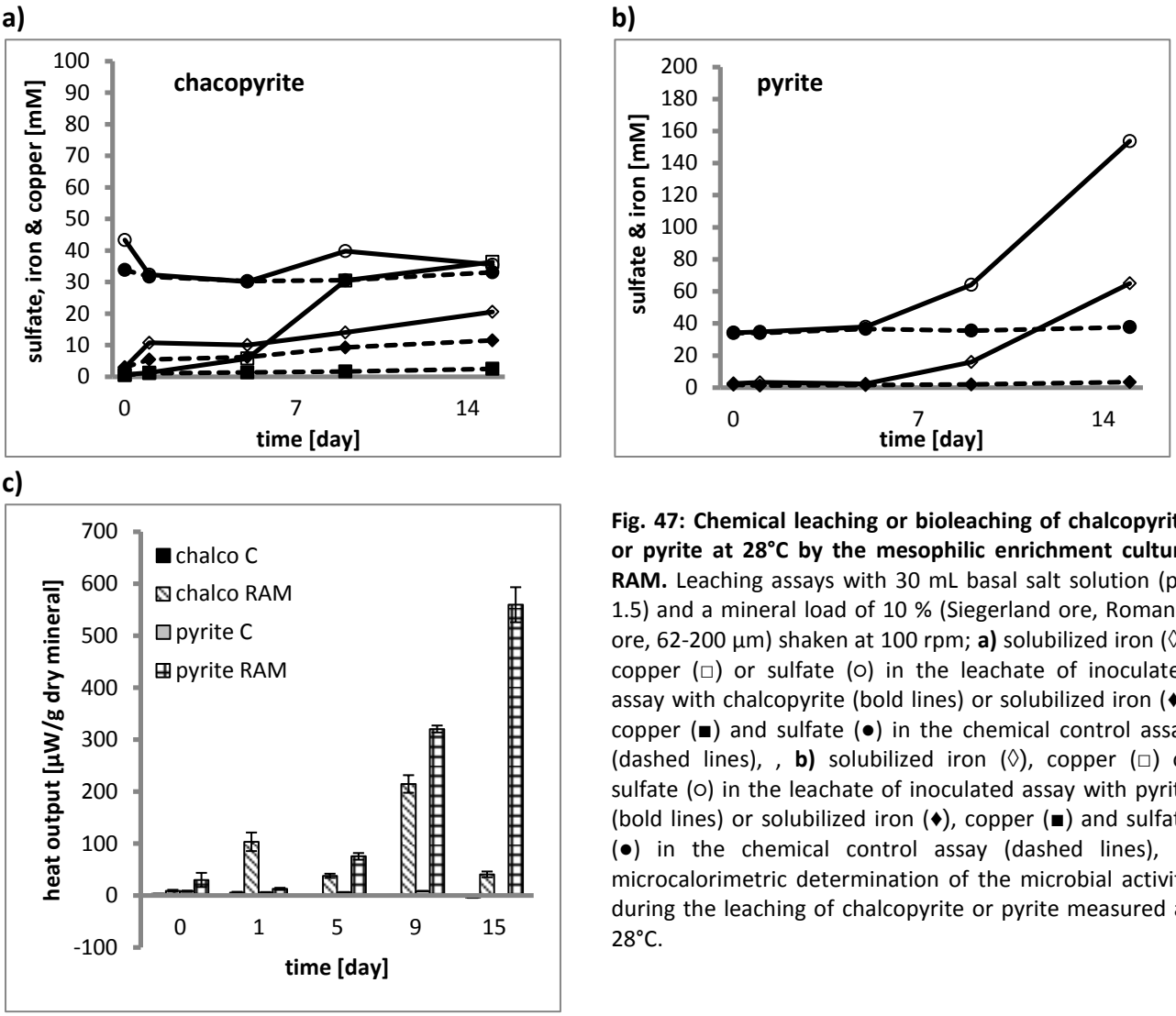

Fig. 47: Chemical leaching or bioleaching of chalcopyrite or pyrite at 28°C by the mesophilic enrichment culture RAM. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (Siegerland ore, Romania ore, 62-200 µm) shaken at 100 rpm; **a)** solubilized iron (◊), copper (□) or sulfate (○) in the leachate of inoculated assay with chalcopyrite (bold lines) or solubilized iron (♦), copper (■) and sulfate (●) in the chemical control assay (dashed lines), , **b)** solubilized iron (◊), copper (□) or sulfate (○) in the leachate of inoculated assay with pyrite (bold lines) or solubilized iron (♦), copper (■) and sulfate (●) in the chemical control assay (dashed lines), **c)** microcalorimetric determination of the microbial activity during the leaching of chalcopyrite or pyrite measured at 28°C.

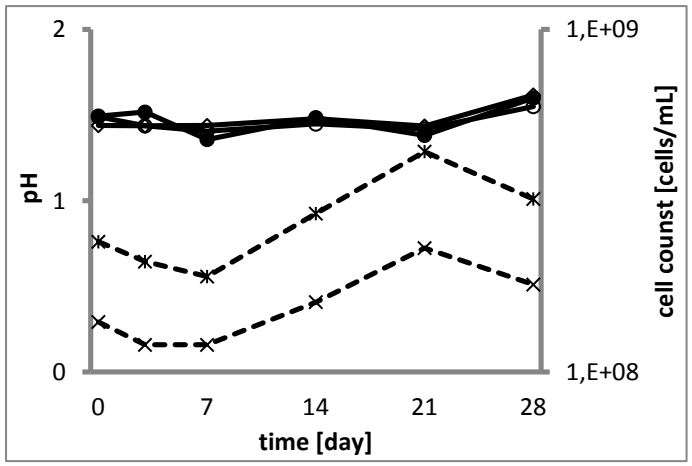

Fig. 48: pH and cell counts during chemical leaching or bioleaching of chalcopyrite at 28°C by *Acidithiobacillus ferrooxidans* (ATCC 53993) pre-cultured on sulfur or chalcopyrite. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 2 % (Siegerland ore, 62-200 µm) incubated at 28°C and shaken at 100 rpm; pH of *At. ferrooxidans* (pre-grown on sulfur) assay (○), *At. ferrooxidans* (pre-grown on chalcopyrite) assay (◊); pH of chemical control assay (●), cell counts of *At. ferrooxidans* (pre-grown on sulfur) (x), cell counts of *At. ferrooxidans* (pre-grown on chalcopyrite) (ж).

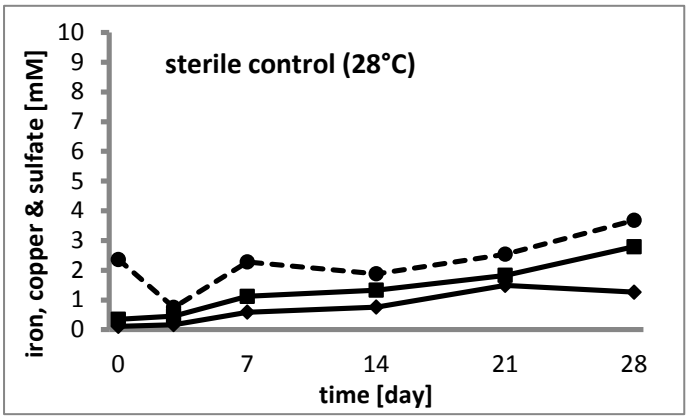

Fig. 49: Chemical leaching of chalcopyrite at 28°C Leaching assay with 50 mL basal salt solution (pH 1.5) and a mineral load of 2 % (Siegerland ore, 62-200 µm shaken at 100 rpm; **a)** solubilized iron (♦), copper (■) or sulfate (●) in the leachate.

Calorimetric measurements of chalcopyrite degradation at 45°C

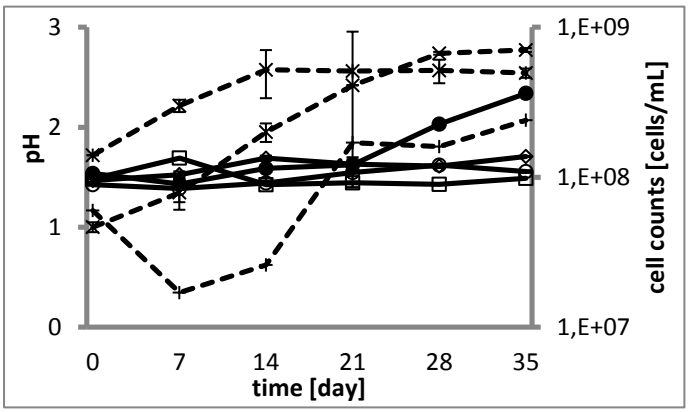

Fig. 50: pH and cell counts during chemical leaching or bioleaching of chalcopyrite at 45°C by the moderate thermophilic enrichment culture AS pre-cultured on sulfur, chalcopyrite or pyrite. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 2 % (Siegerland ore, 62-200 µm) shaken at 100 rpm; pH of AS cultures pre-grown on sulfur (○),chalcopyrite (◊) or on pyrite (□); pH of chemical control assay (●), cell counts of AS pre-grown on sulfur (x), on chalcopyrite (ж) or on pyrite (+).

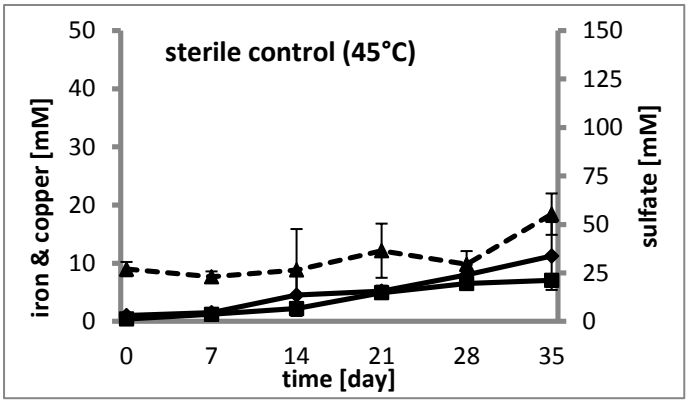

Fig. 51: Chemical leaching of chalcopyrite at 45°C. Leaching assays with 30 mL basal salt solution and a mineral load of 2 % (Siegerland ore, 62-200 µm) shaken at 100 rpm; solubilized iron (♦), copper (■) and sulfate (●) in the leachate.

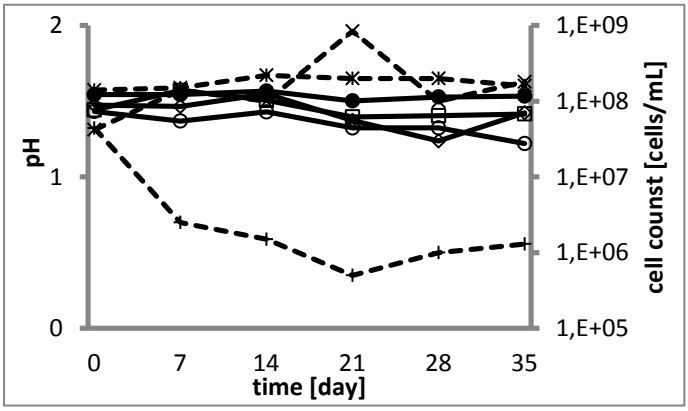

Fig. 52: pH and cell counts during chemical leaching or bioleaching of pyrite at 45°C by the moderate thermophilic enrichment culture AS pre-cultured on sulfur, chalcopyrite or pyrite. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 2 % (Romania ore, 62-200 µm) shaken at 100 rpm; pH of AS pre-grown on sulfur (○), pH of AS pre-grown on chalcopyrite (◊), pH of AS pre-grown on pyrite (□); pH of chemical control assay (●); cell counts of AS pre-grown on sulfur (x), on chalcopyrite (ж) or on pyrite (+).

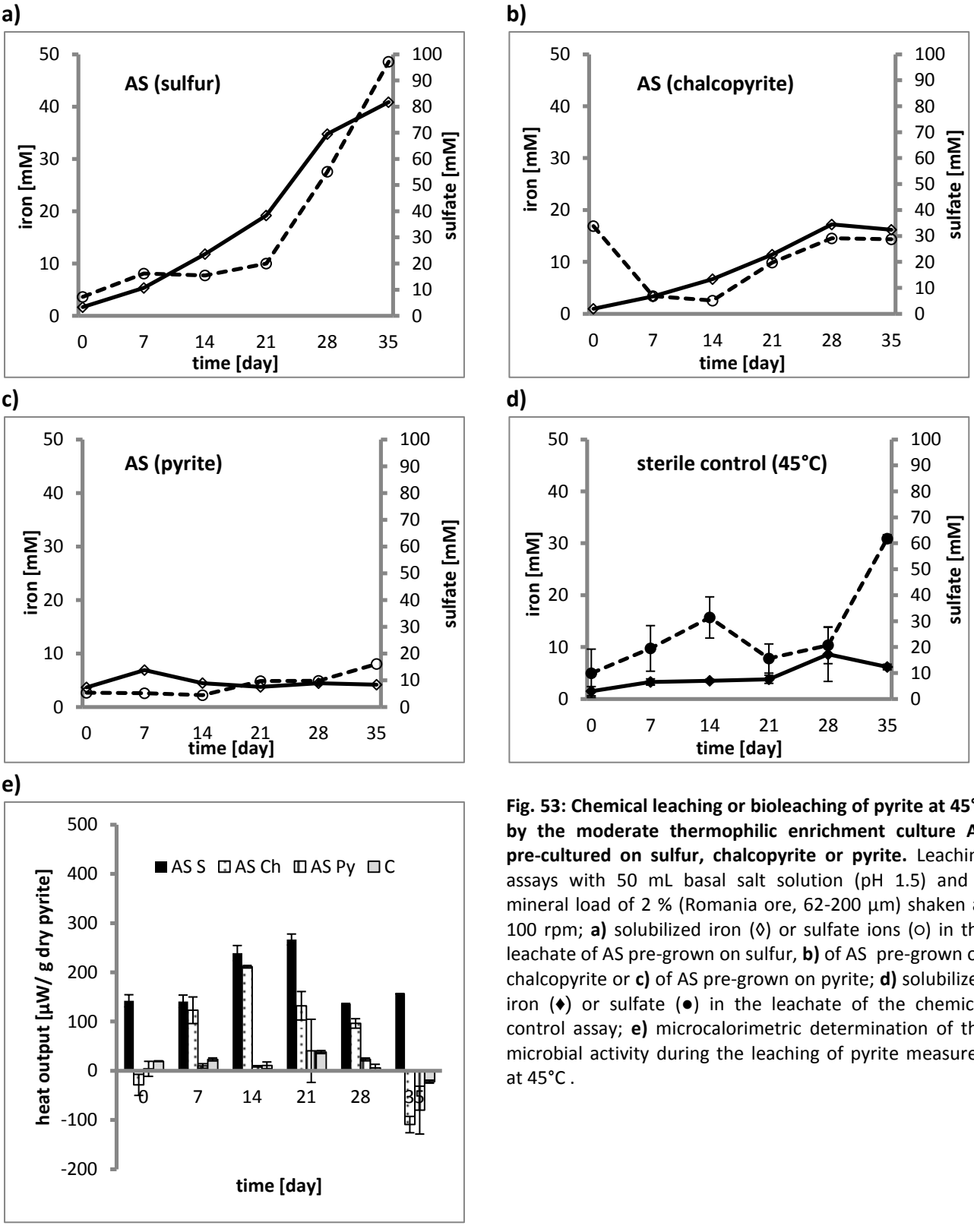

Fig. 53: Chemical leaching or bioleaching of pyrite at 45°C by the moderate thermophilic enrichment culture AS pre-cultured on sulfur, chalcopyrite or pyrite. Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 2 % (Romania ore, 62-200 µm) shaken at 100 rpm; **a)** solubilized iron (◊) or sulfate ions (○) in the leachate of AS pre-grown on sulfur, **b)** of AS pre-grown on chalcopyrite or **c)** of AS pre-grown on pyrite; **d)** solubilized iron (♦) or sulfate (●) in the leachate of the chemical control assay; **e)** microcalorimetric determination of the microbial activity during the leaching of pyrite measured at 45°C .

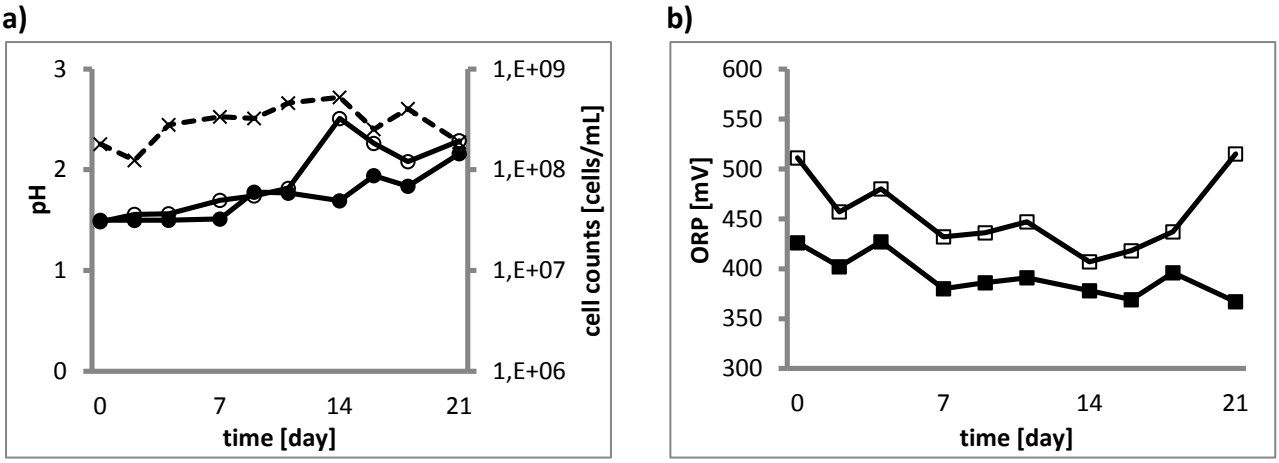

Fig. 54: a) pH, cell counts or b) ORP during chemical leaching or bioleaching of chalcopyrite at 45°C by the moderate thermophilic enrichment culture AS. Leaching assays with 30 mL (pH 1.5) basal salt solution and a mineral load of 10 % (Siegerland ore, 62-200 µm) shaken at 100 rpm; pH of AS assay (○), pH of chemical control assay (●); cell counts of AS assay (x); ORP of AS (□) or chemical control assay (■).

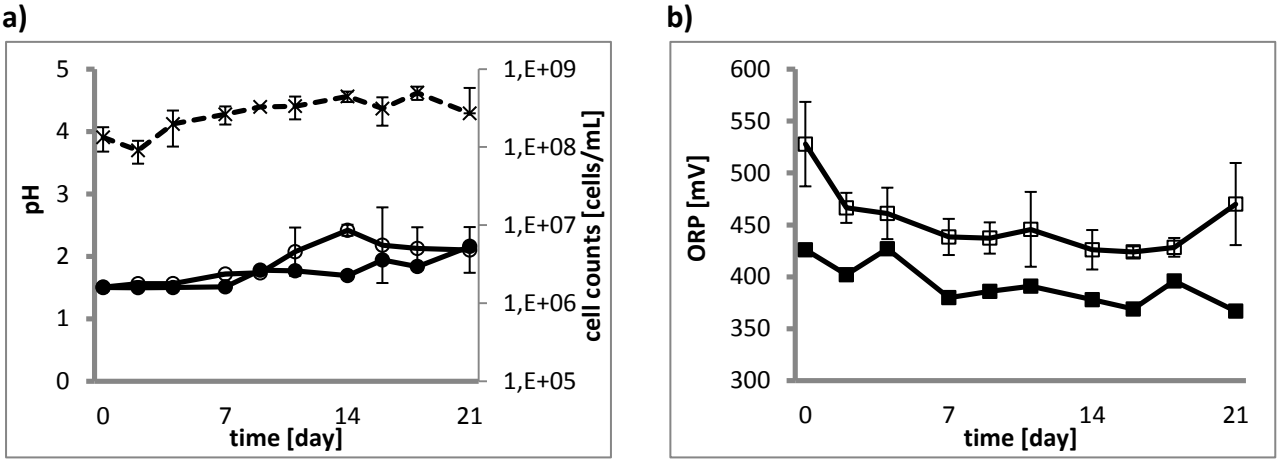

Fig. 55: a) pH, cell counts or b) ORP during chemical leaching or bioleaching of chalcopyrite at 45°C by the chalcopyrite adapted moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (Siegerland ore, 62-200 µm) shaken at 100 rpm; pH of AS assay (○), pH of chemical control assay (●); cell counts of AS assay (x); ORP of AS (□) or chemical control assay (■).

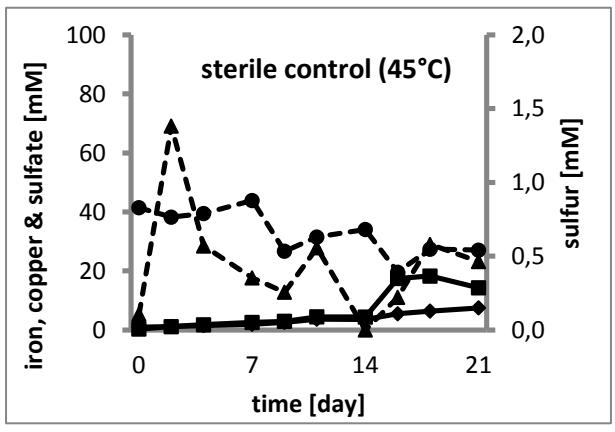

Fig. 56: Chemical leaching of chalcopyrite at 45°C. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (Siegerland ore, 62-200 µm) shaken at 100 rpm; solubilized iron (♦), copper (■), sulfate (●) or sulfur (▲) in the leachate.

Calorimetric measurements on chalcopyrite degradation at 65°C

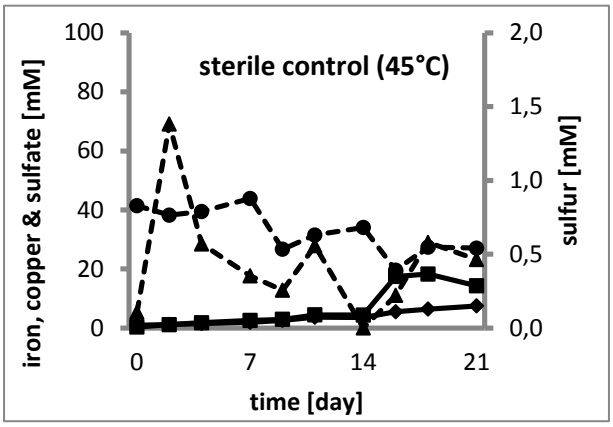

Figure 56. Chemical leaching of chalcopyrite at 45°C. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (Siegerland ore, 62-200 µm) shaken at 100 rpm; solubilized iron (♦), copper (■), sulfate (●) or sulfur (▲) in the leachate.

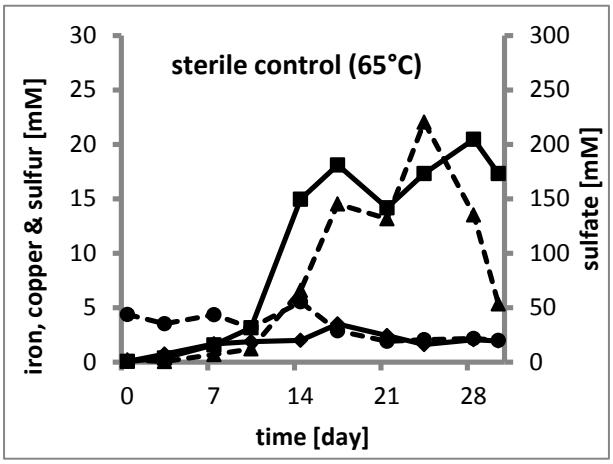

Fig. 58: Chemical leaching of chalcopyrite at 65°C. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (Siegerland ore, 62-200 µm) shaken at 100 rpm; **a)** solubilized iron (♦), copper (■), sulfate (●) or sulfur (▲) in the leachate.

Calorimetric measurements of chalcocite and covellite degradation

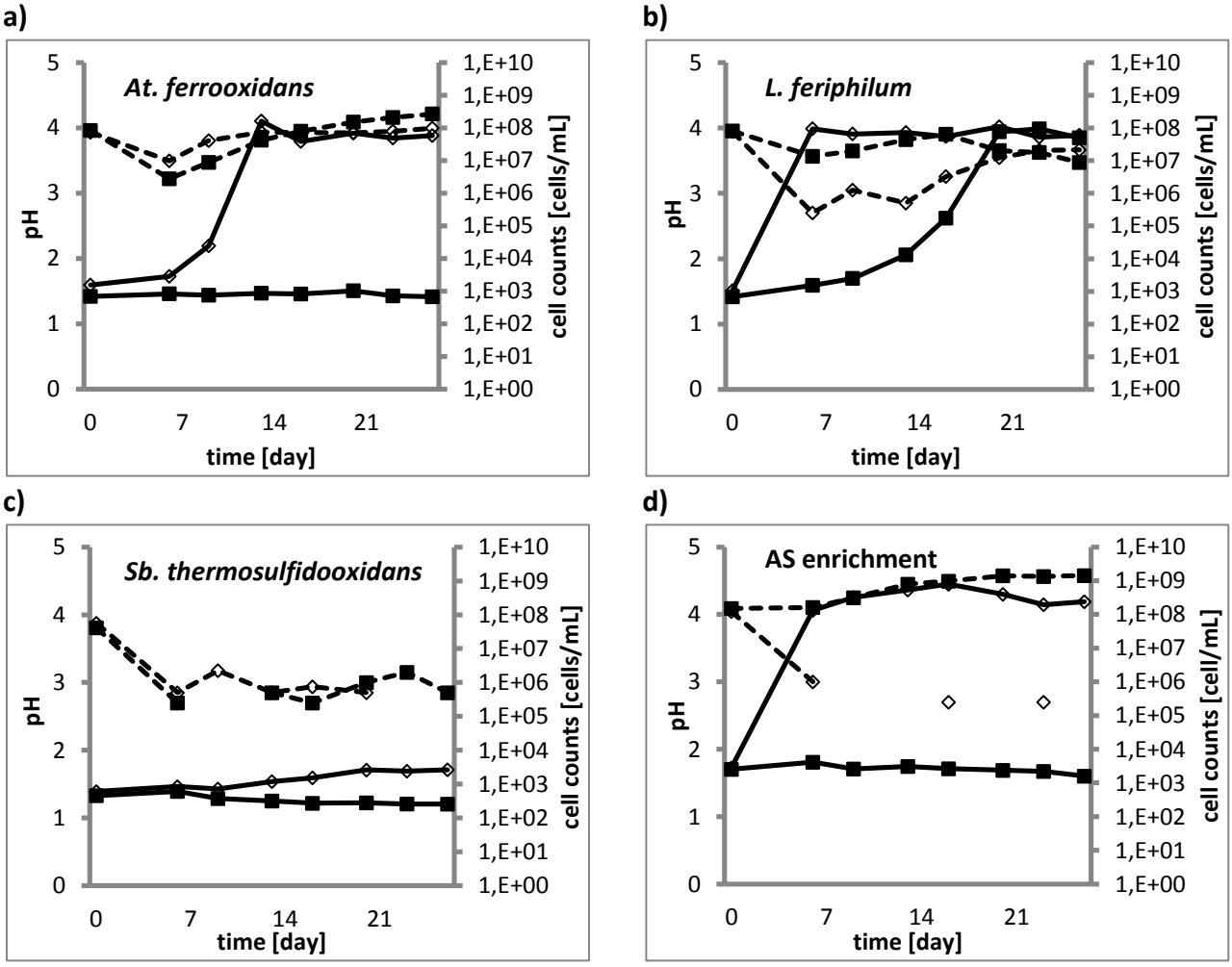

Fig. 59: pH and cell counts of a) *At. ferrooxidans* at 28°C, b) *L. ferriphilum* at 45°C, c) *Sb. thermosulfidooxidans* at 45°C or d) the moderate thermophilic enrichment culture AS at 45°C during the leaching of chlacocite or covellite. Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 1% mineral (> 50 µm) shaken at 120 rpm; pH (bold line) and cell counts (dashed line) in (◊) chalcocite or (■) covellite assays.

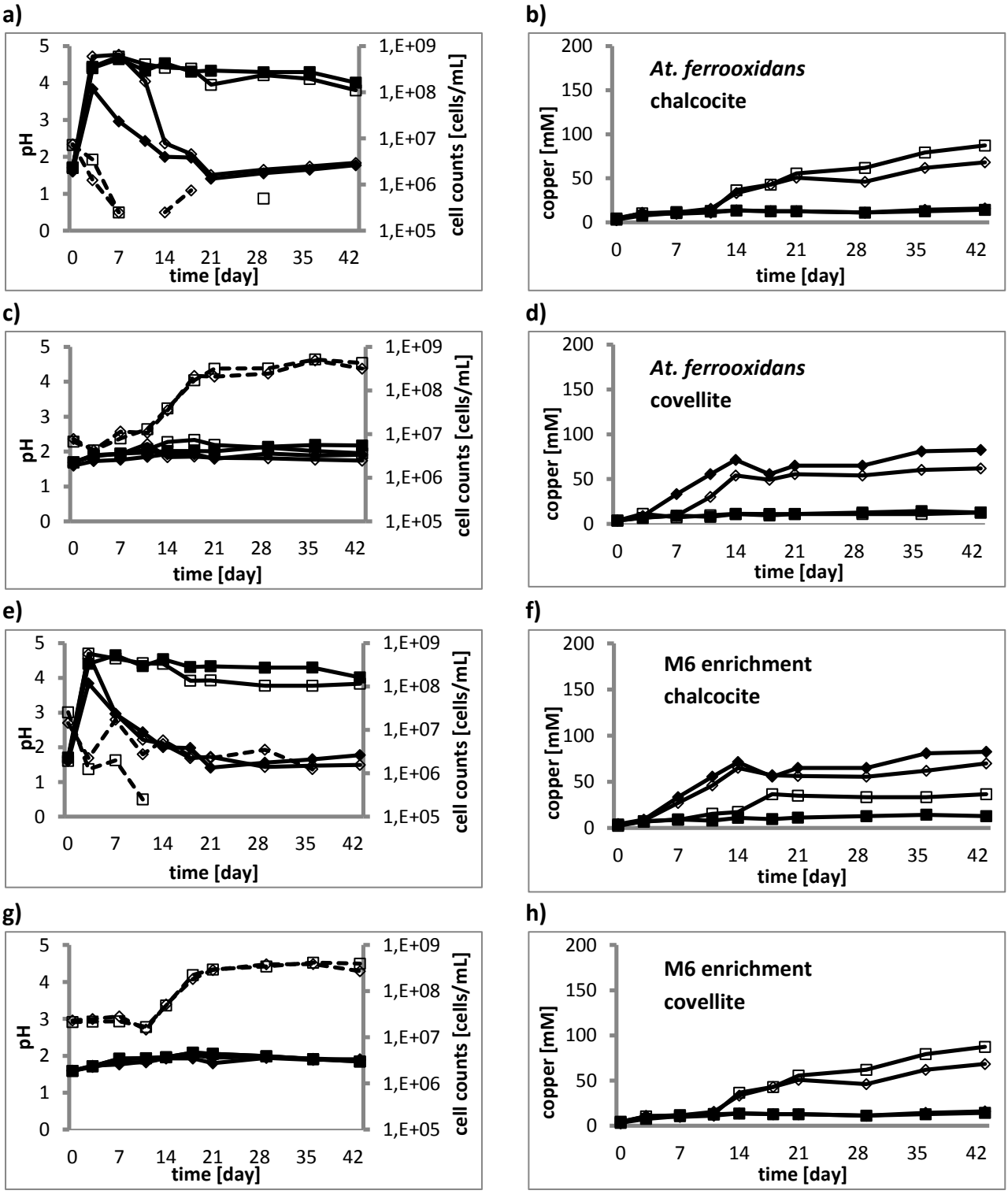

Fig. 60: pH, cell counts or copper solubilization during chemical leaching or bioleaching of chalcocite or covellite at 28°C by *At. ferrooxidans* (a, b, c and d) or the mesophilic enrichment culture M6 (e, f, g and h) with or without pH adjustment. Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 10 % (> 50 µm) shaken at 120 rpm; pH was adjusted to a value between 1.5 and 2 with conc. sulfuric acid; **a)** and **e)** pH (bold lines) and cell counts (dashed lines) of chalcocite leaching; **c)** and **g)** pH (bold lines) and cell counts (dashed lines) of covellite leaching, inoculated assays with (◊) or without pH adjustment (□), sterile control assays with (♦) or without pH adjustment (■); **b)** and **f)** solubilized copper in the leachate of chalcocite assays; **d)** and **h)** solubilized copper in the leachate of covellite assays with (◊) or without pH adjustment (□), sterile control assays with (♦) or without pH adjustment (■).

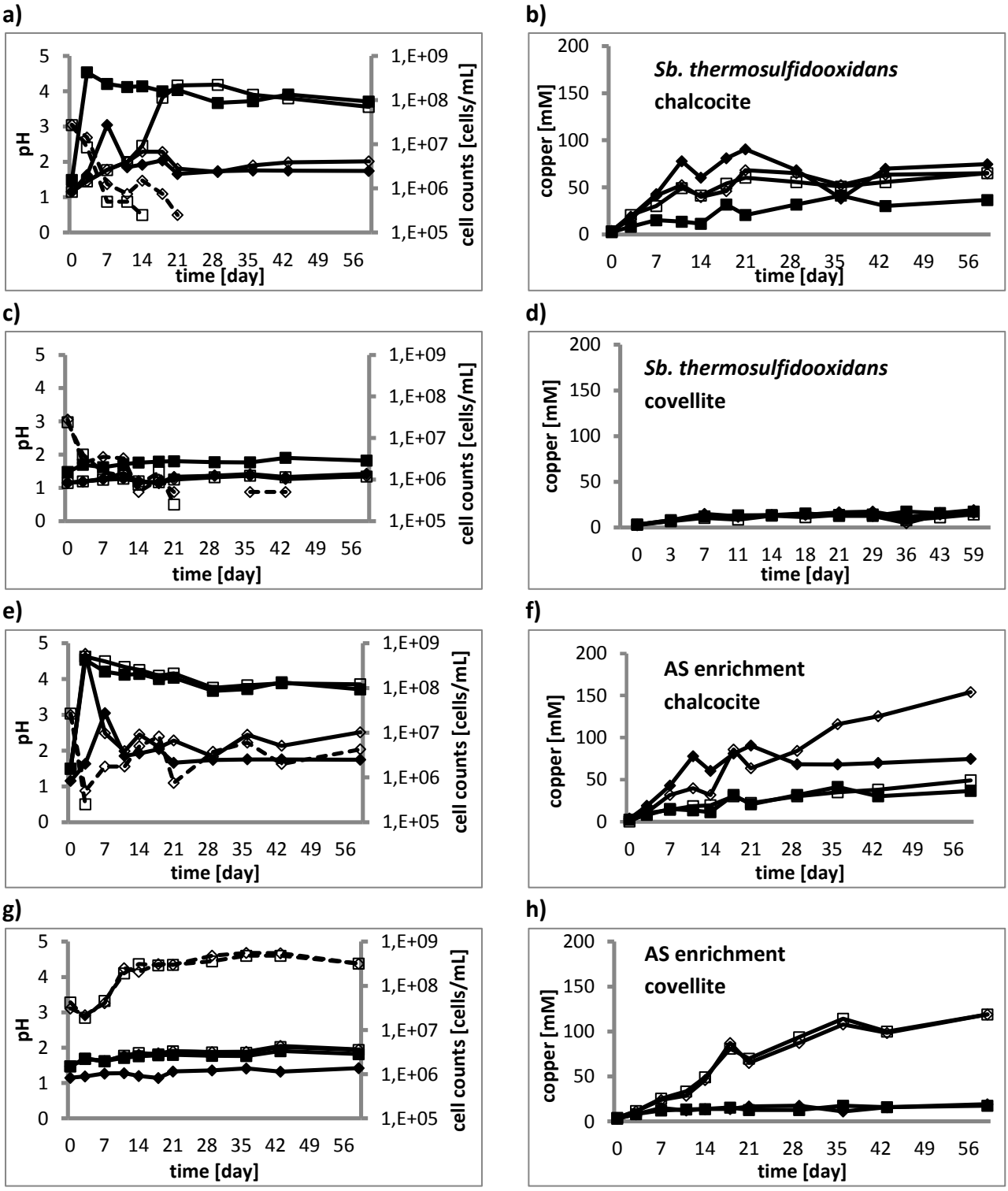

Fig. 61: pH, cell counts and copper solubilization during chemical leaching or bioleaching of chalcocite or covellite at 45°C by *Sb. thermosulfidooxdans* (a, b, c and d) or the moderate thermophilic enrichment culture AS (e, f, g and h) with or without pH adjustment. Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 10 % (> 50 µm) shaken at 120 rpm; pH was adjusted to a value between 1.5 and 2 with conc. sulfuric acid; **a)** and **e)** pH (bold lines) and cell counts (dashed lines) of chalcocite leaching; **c)** and **g)** pH (bold lines) and cell counts (dashed lines) of covellite leaching, inoculated assays with (◊) or without pH adjustment (□), sterile control assays with (♦) or without pH adjustment (■); **b)** and **f)** solubilized copper in the leachate of chalcocite assays; **d)** and **h)** solubilized copper in the leachate of covellite assays with (◊) or without pH adjustment (□), sterile control assays with (♦) or without pH adjustment (■).

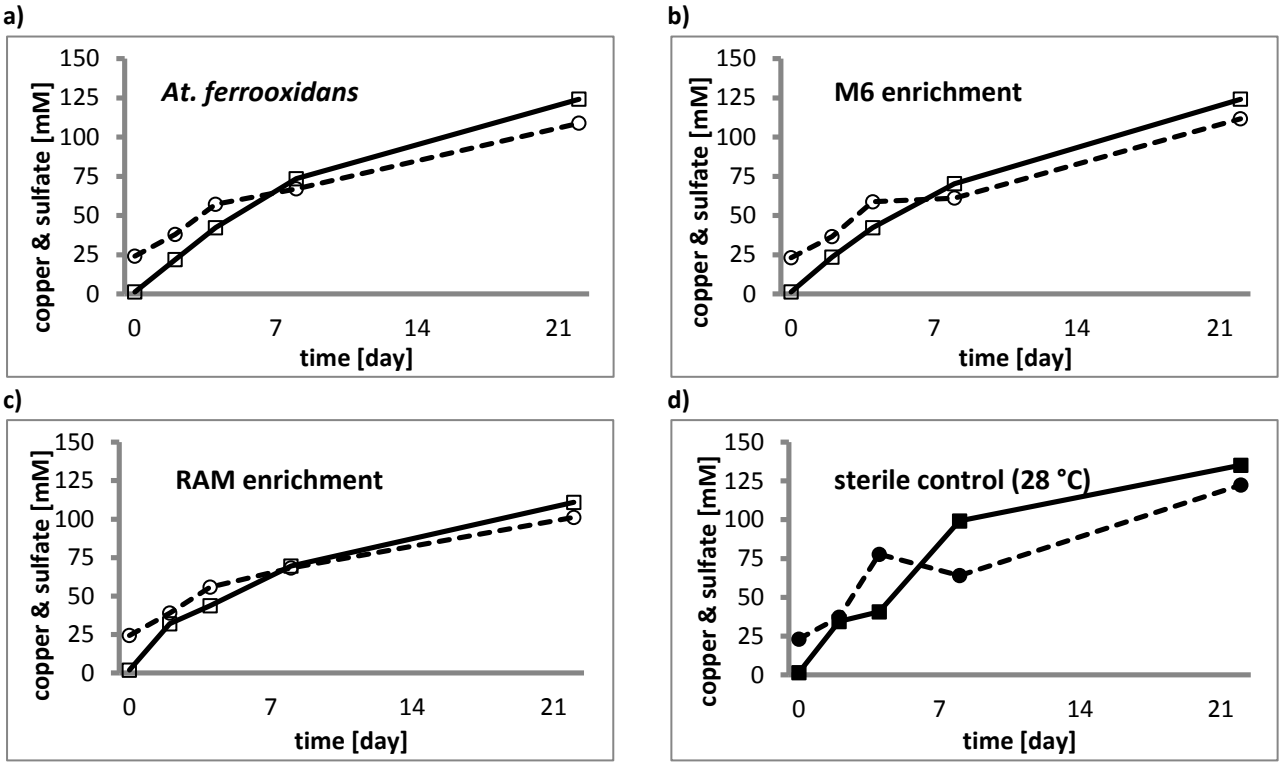

Fig. 62: Chemical leaching or bioleaching of chalcocite at 28°C by a) *At. ferrooxidans* or the mesophilic enrichment cultures b) M6 or c) RAM. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (62-200 µm) shaken at 100 rpm; copper (□), sulfate (○); **d)** solubilized copper (■) or sulfate (●) in the leachate of the chemical control assay.

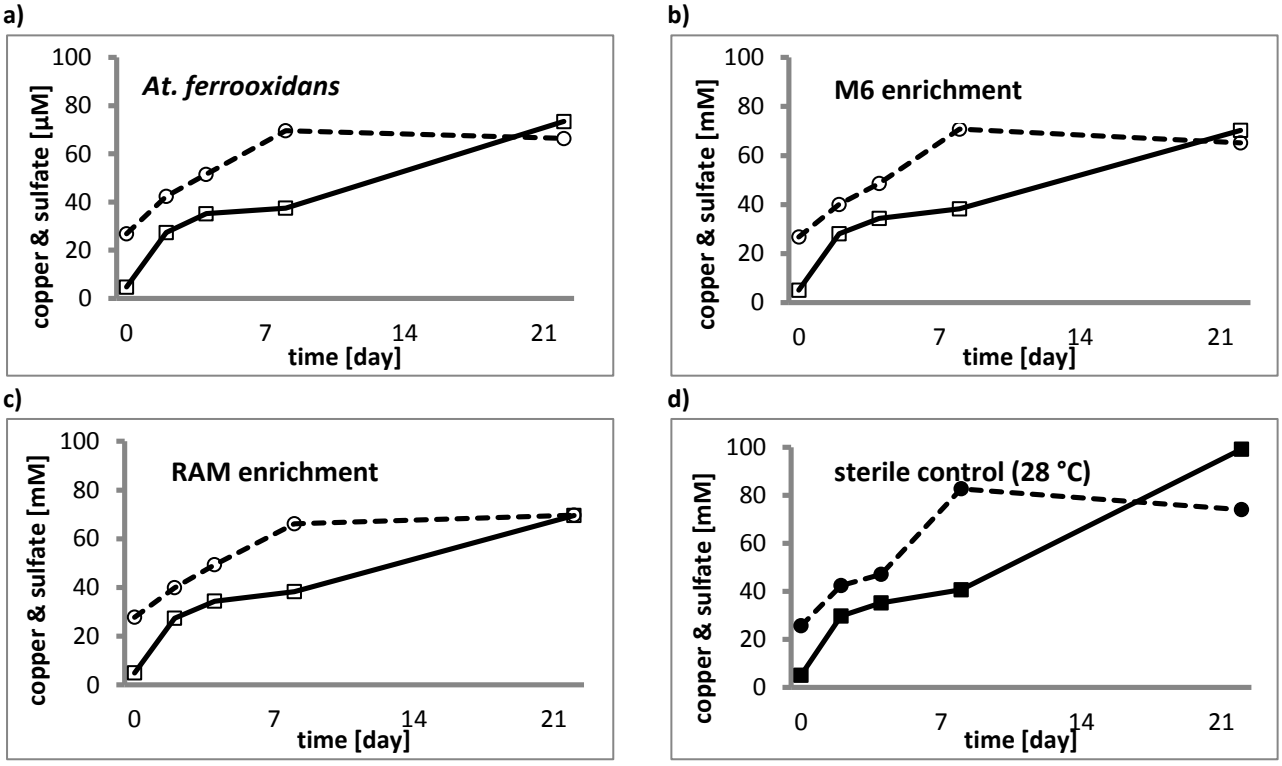

Fig. 63: Chemical leaching or bioleaching of covellite at 28°C by a) *At. ferrooxidans* or the mesophilic enrichment cultures b) M6 or c) RAM. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (62-200 µm) shaken at 100 rpm; copper (□) and sulfate (○); **d)** solubilized copper (■) or sulfate (●) in the leachate of the chemical control assay.

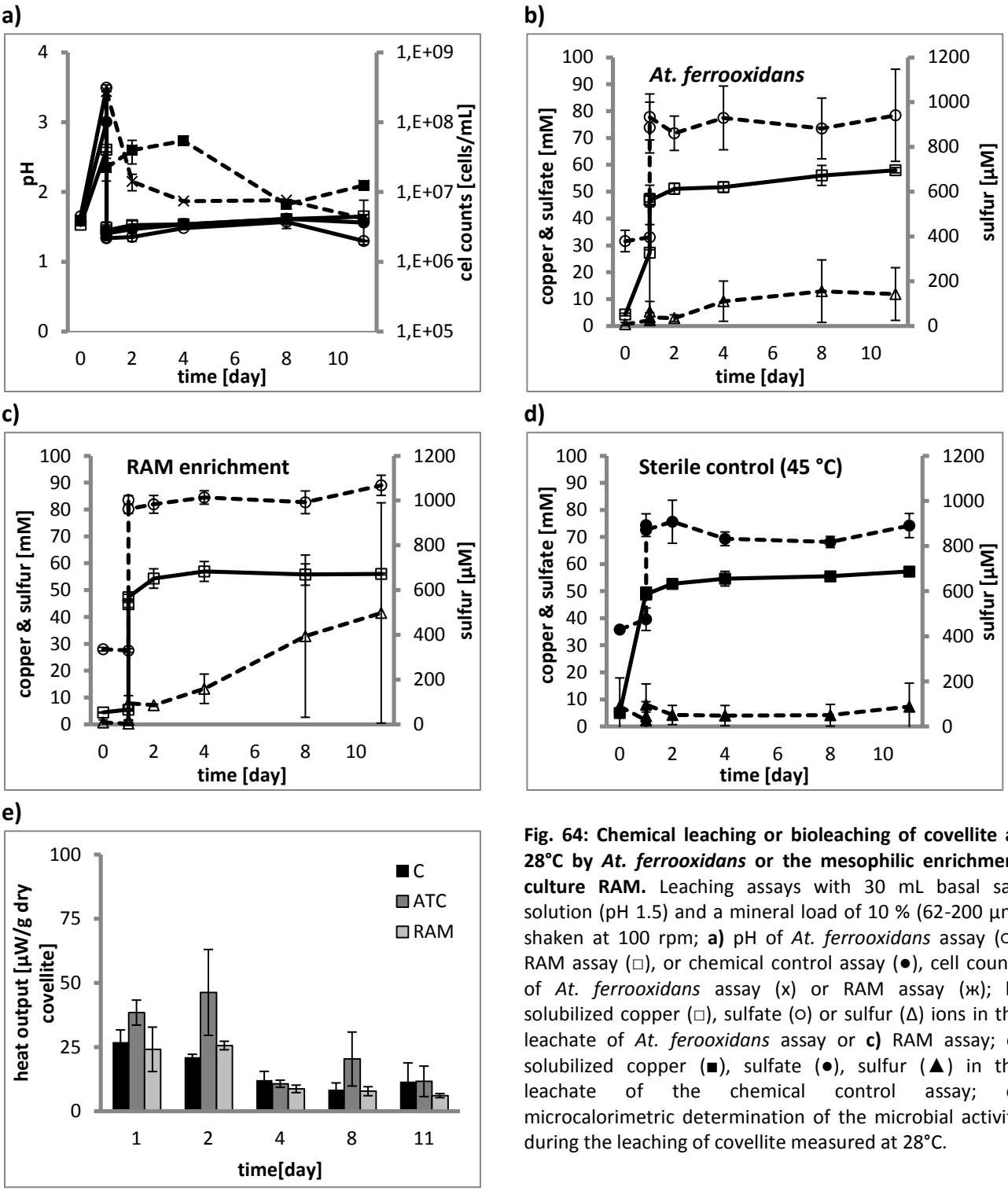

Fig. 64: Chemical leaching or bioleaching of covellite at 28°C by *At. ferrooxidans* or the mesophilic enrichment culture RAM. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (62-200 µm) shaken at 100 rpm; **a)** pH of *At. ferrooxidans* assay (○), RAM assay (□), or chemical control assay (●), cell counts of *At. ferrooxidans* assay (x) or RAM assay (ж); **b)** solubilized copper (□), sulfate (○) or sulfur (Δ) ions in the leachate of *At. ferooxidans* assay or **c)** RAM assay; **e)** solubilized copper (■), sulfate (●), sulfur (▲) in the leachate of the chemical control assay; **e)** microcalorimetric determination of the microbial activity during the leaching of covellite measured at 28°C.

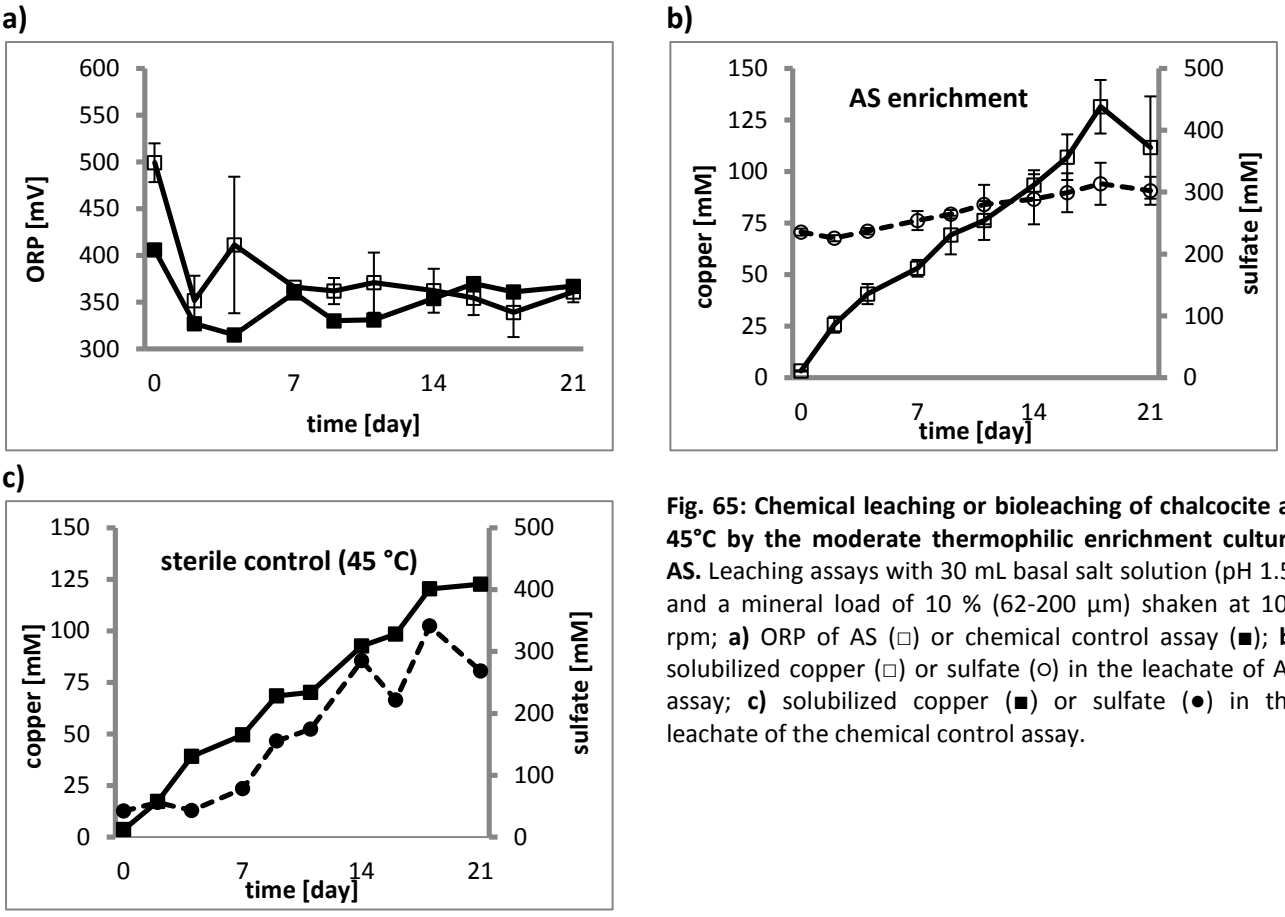

Fig. 65: Chemical leaching or bioleaching of chalcocite at 45°C by the moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (62-200 µm) shaken at 100 rpm; **a)** ORP of AS (□) or chemical control assay (■); **b)** solubilized copper (□) or sulfate (○) in the leachate of AS assay; **c)** solubilized copper (■) or sulfate (●) in the leachate of the chemical control assay.

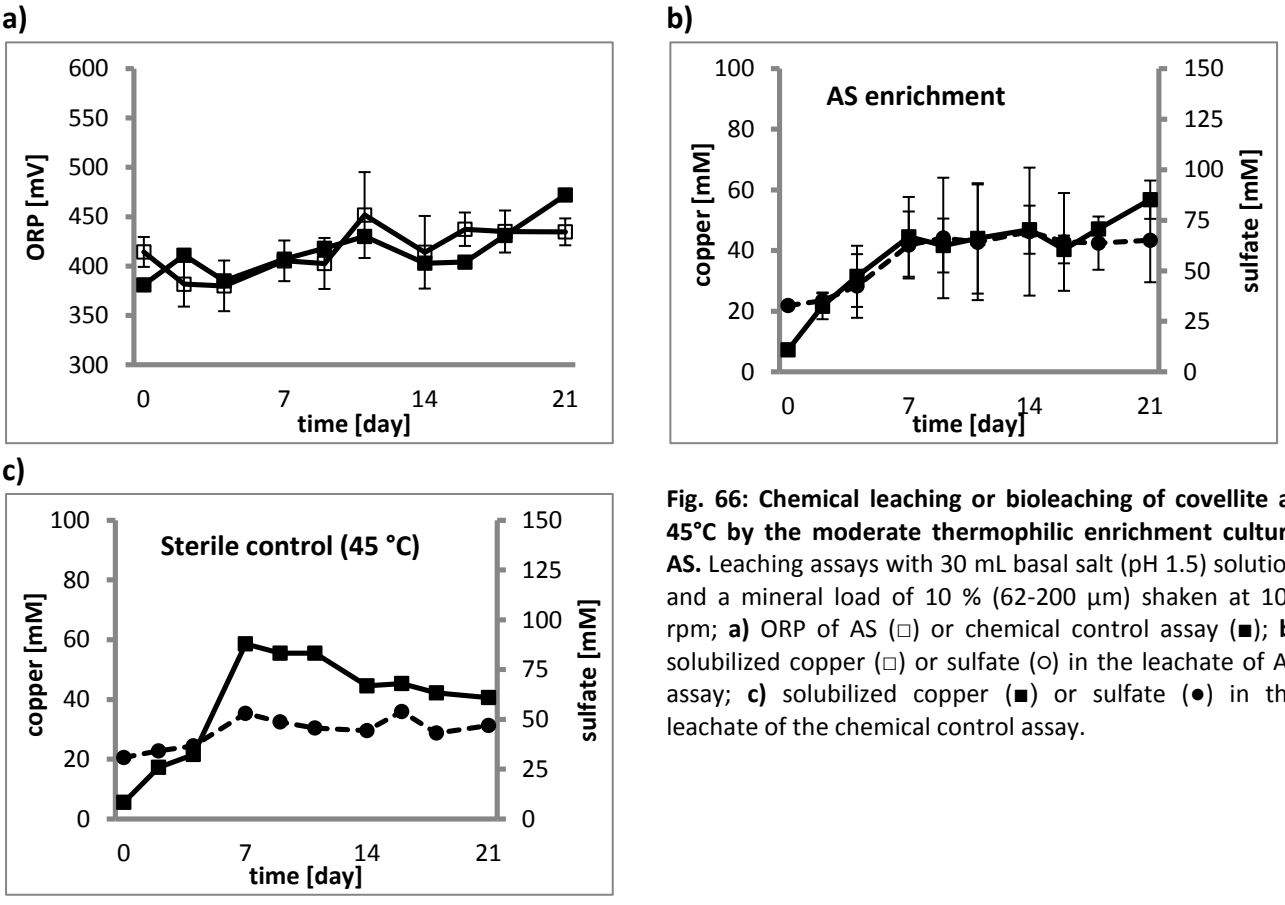

Fig. 66: Chemical leaching or bioleaching of covellite at 45°C by the moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt (pH 1.5) solution and a mineral load of 10 % (62-200 µm) shaken at 100 rpm; **a)** ORP of AS (□) or chemical control assay (■); **b)** solubilized copper (□) or sulfate (○) in the leachate of AS assay; **c)** solubilized copper (■) or sulfate (●) in the leachate of the chemical control assay.

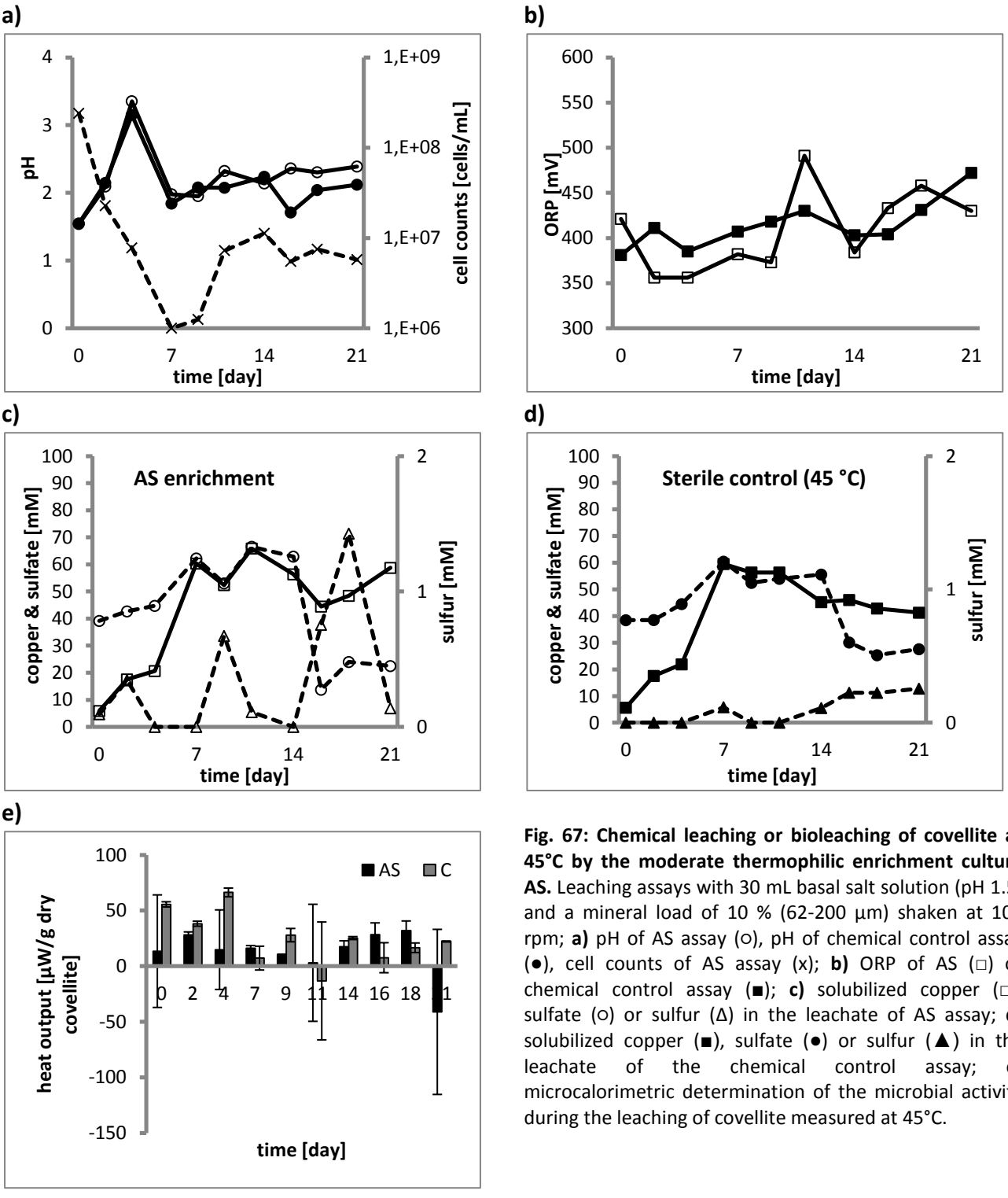

Fig. 67: Chemical leaching or bioleaching of covellite at 45°C by the moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % (62-200 µm) shaken at 100 rpm; **a)** pH of AS assay (○), pH of chemical control assay (●), cell counts of AS assay (x); **b)** ORP of AS (□) or chemical control assay (■); **c)** solubilized copper (□), sulfate (○) or sulfur (Δ) in the leachate of AS assay; **d)** solubilized copper (■), sulfate (●) or sulfur (▲) in the leachate of the chemical control assay; **e)** microcalorimetric determination of the microbial activity during the leaching of covellite measured at 45°C.

Microscopic observations during chalcopyrite bioleaching

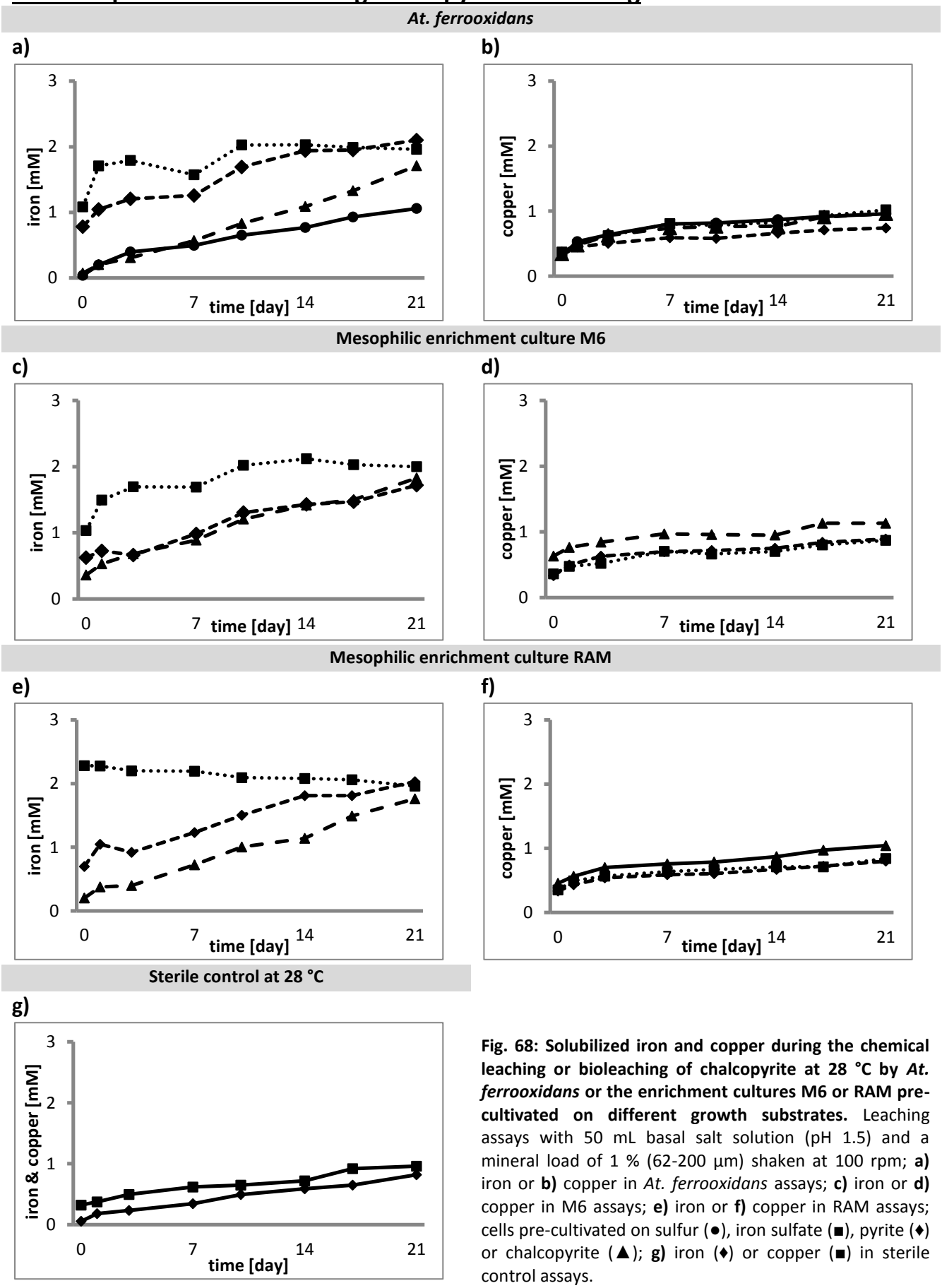

Fig. 68: Solubilized iron and copper during the chemical leaching or bioleaching of chalcopyrite at 28 °C by *At. ferrooxidans* or the enrichment cultures M6 or RAM pre-cultivated on different growth substrates. Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 1 % (62-200 µm) shaken at 100 rpm; **a)** iron or **b)** copper in *At. ferrooxidans* assays; **c)** iron or **d)** copper in M6 assays; **e)** iron or **f)** copper in RAM assays; cells pre-cultivated on sulfur (●), iron sulfate (■), pyrite (♦) or chalcopyrite (▲); **g)** iron (♦) or copper (■) in sterile control assays.

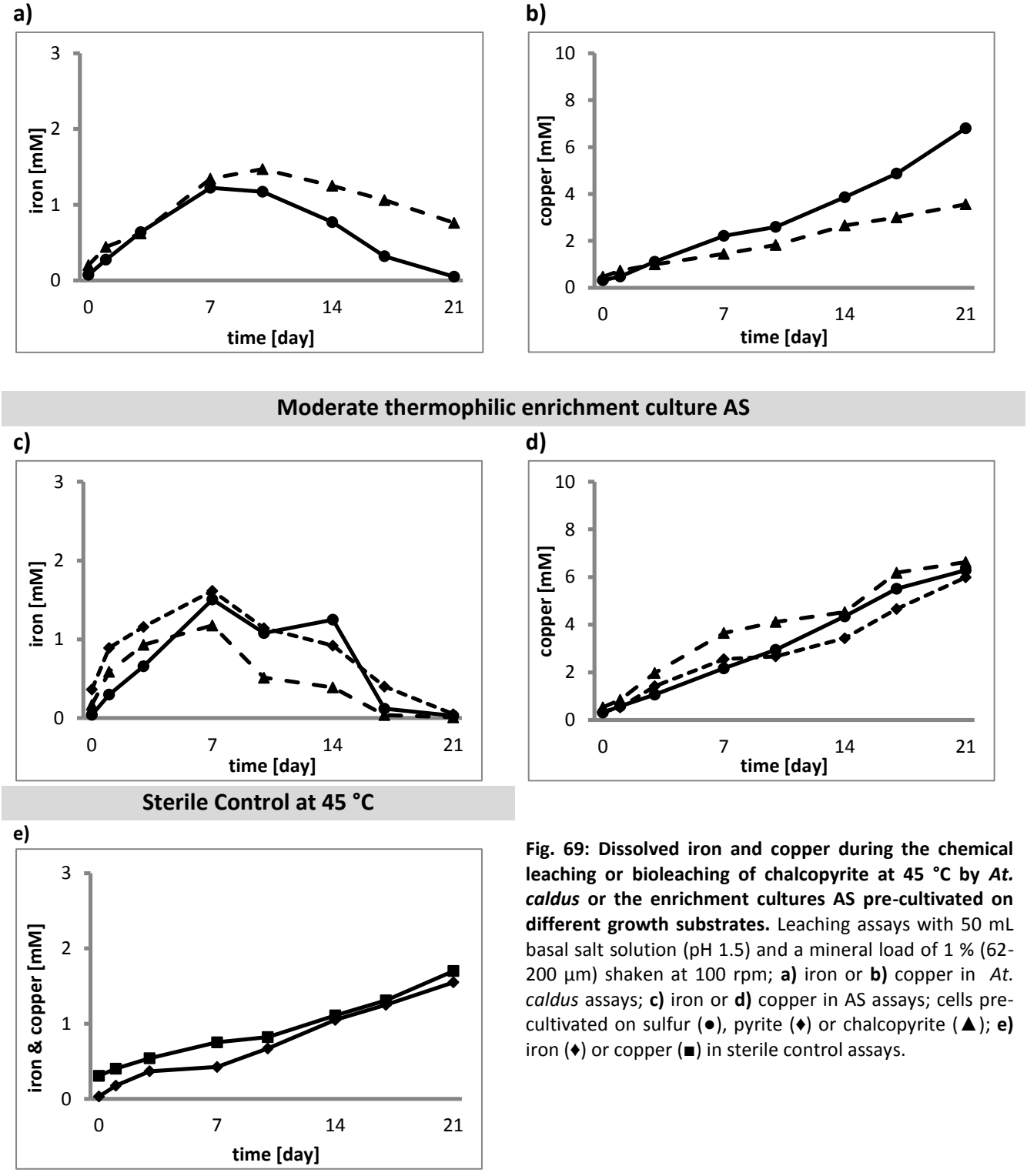

Fig. 69: Dissolved iron and copper during the chemical leaching or bioleaching of chalcopyrite at 45 °C by *At. caldus* or the enrichment cultures AS pre-cultivated on different growth substrates. Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 1 % (62-200 µm) shaken at 100 rpm; **a)** iron or **b)** copper in *At. caldus* assays; **c)** iron or **d)** copper in AS assays; cells pre-cultivated on sulfur (●), pyrite (♦) or chalcopyrite (▲); **e)** iron (♦) or copper (■) in sterile control assays.

Cuprous copper and copper sulfide bioleaching

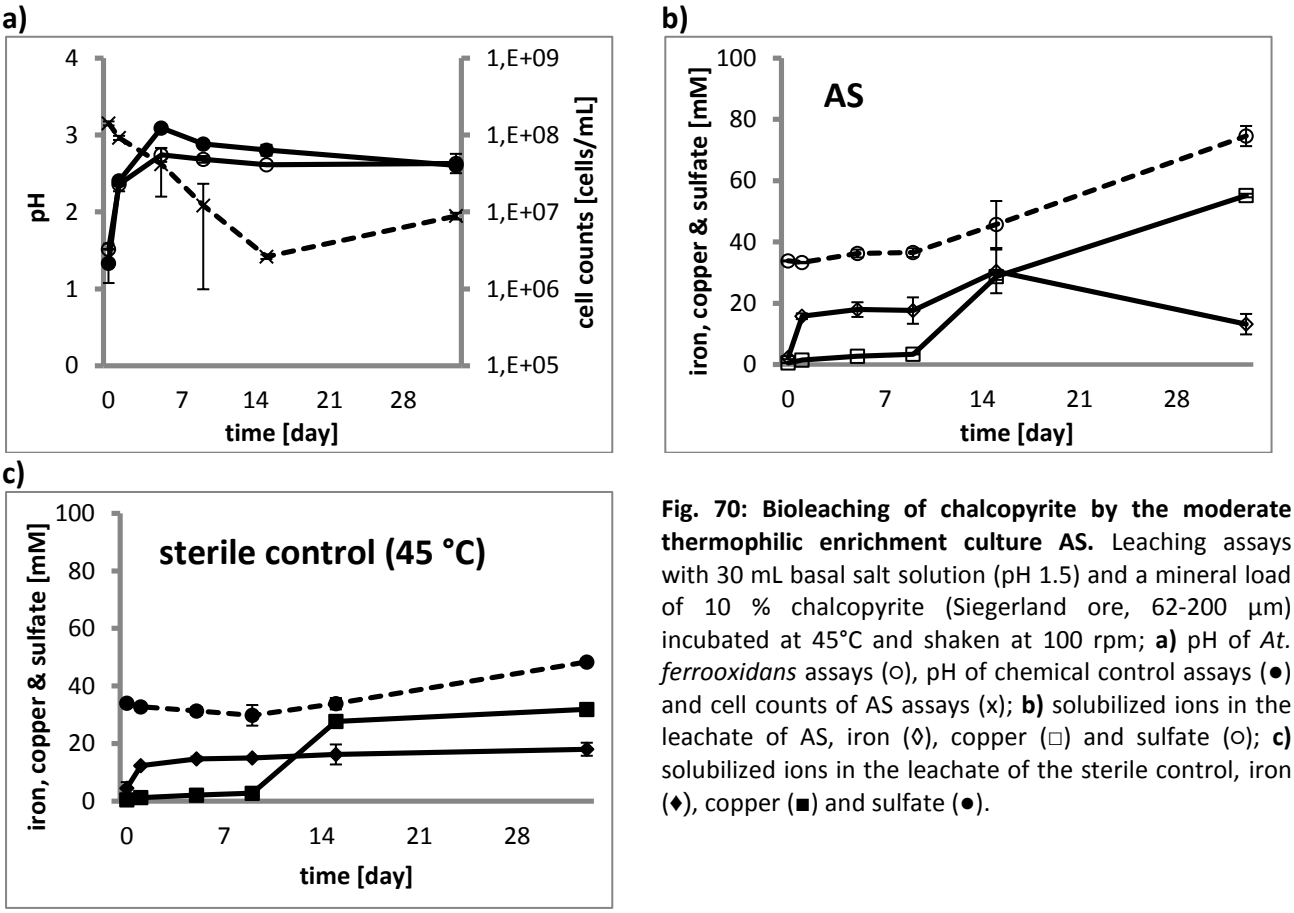

Fig. 70: Bioleaching of chalcopyrite by the moderate thermophilic enrichment culture AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 45°C and shaken at 100 rpm; **a)** pH of *At. ferrooxidans* assays (○), pH of chemical control assays (●) and cell counts of AS assays (x); **b)** solubilized ions in the leachate of AS, iron (◊), copper (□) and sulfate (○); **c)** solubilized ions in the leachate of the sterile control, iron (♦), copper (■) and sulfate (●).

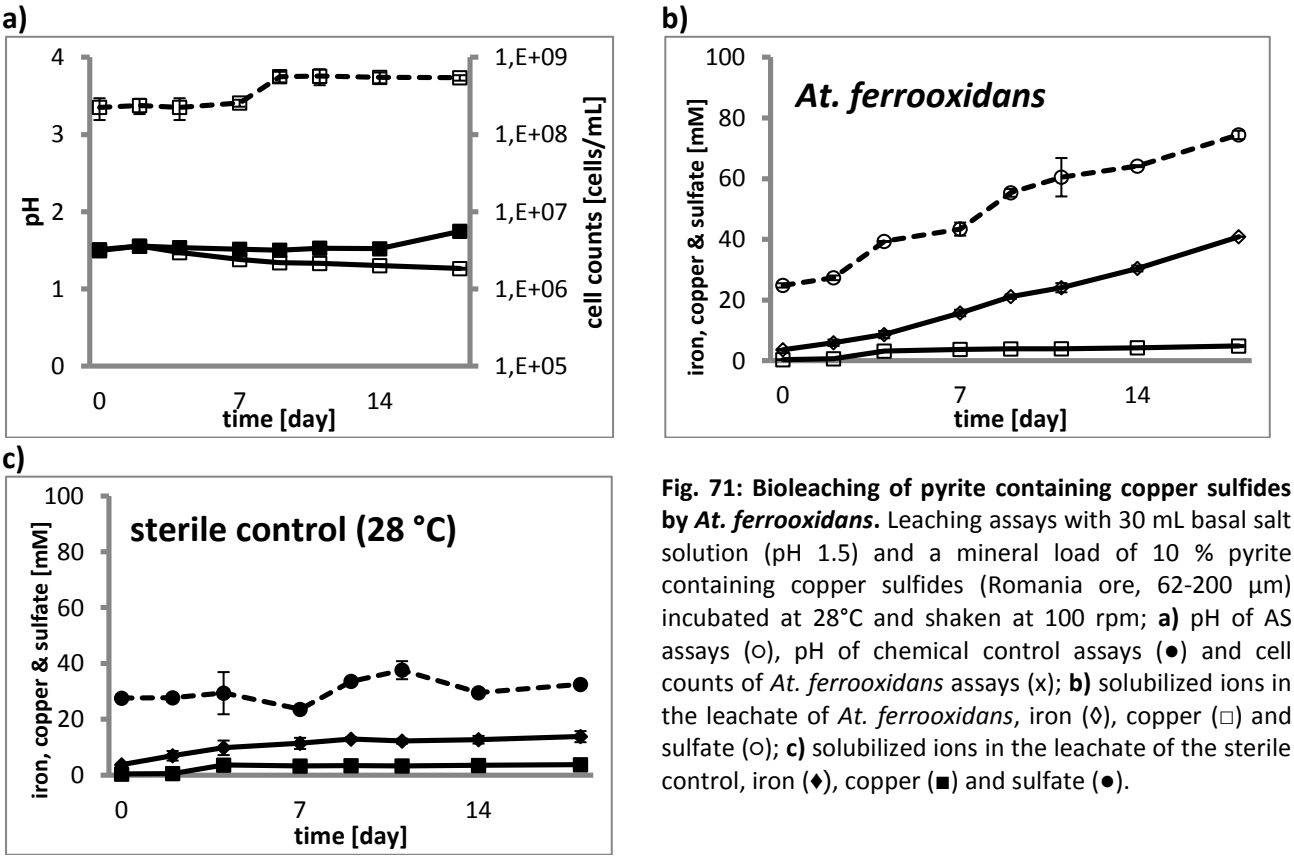

Fig. 71: Bioleaching of pyrite containing copper sulfides by *At. ferrooxidans*. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % pyrite containing copper sulfides (Romania ore, 62-200 µm) incubated at 28°C and shaken at 100 rpm; **a)** pH of AS assays (○), pH of chemical control assays (●) and cell counts of *At. ferrooxidans* assays (x); **b)** solubilized ions in the leachate of *At. ferrooxidans*, iron (◊), copper (□) and sulfate (○); **c)** solubilized ions in the leachate of the sterile control, iron (♦), copper (■) and sulfate (●).

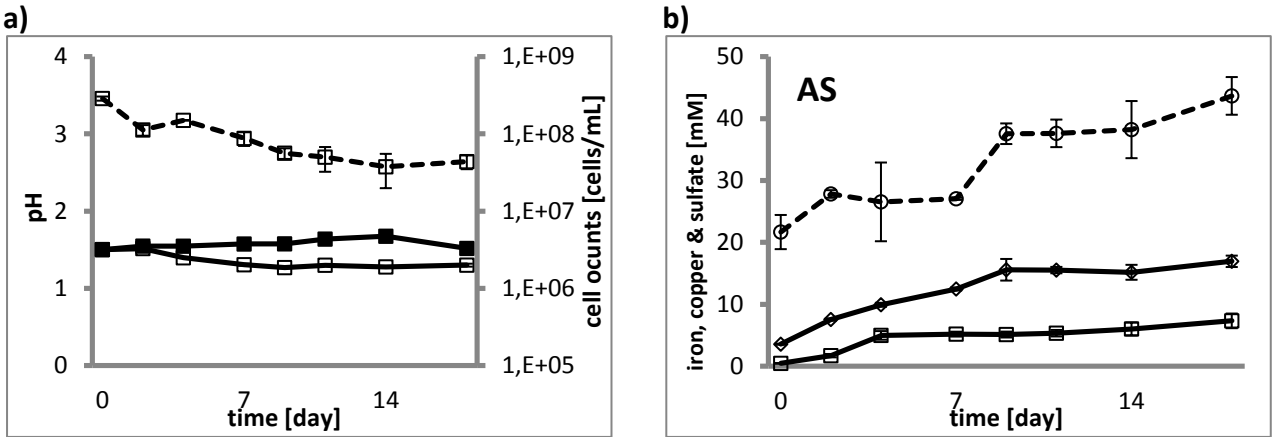

Fig. 72: Bioleaching of pyrite containing copper sulfides by the moderate thermophilic enrichment AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % pyrite containing copper sulfides (Romania ore, 62-200 µm) incubated at 45°C and shaken at 100 rpm; **a)** pH of AS assays (○), pH of chemical control assays (●) and cell counts of AS assays (x); **b)** solubilized ions in the leachate of AS, iron (◊), copper (□) and sulfate (○); **c)** solubilized ions in the leachate of the sterile control, iron (♦), copper (■) and sulfate (●).

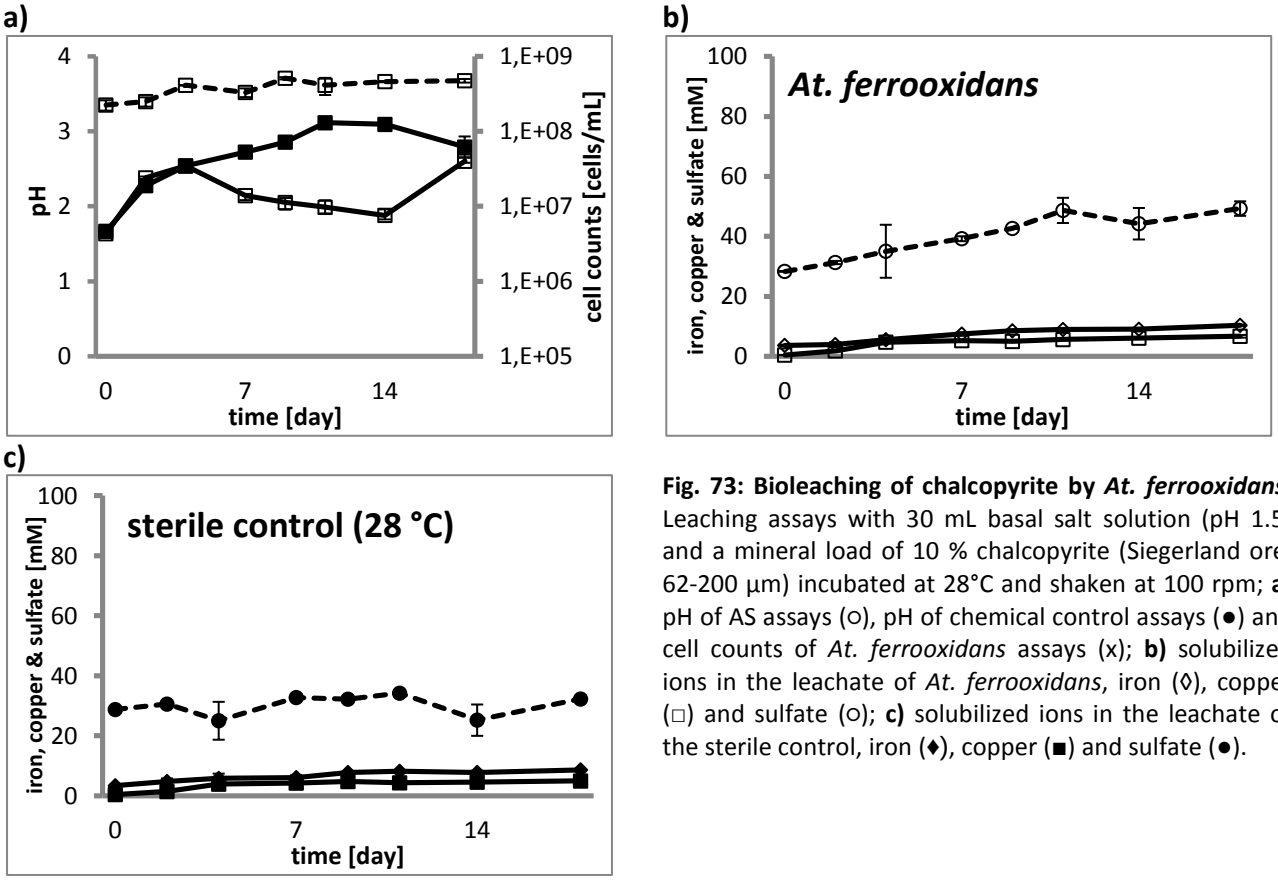

Fig. 73: Bioleaching of chalcopyrite by *At. ferrooxidans*. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 28°C and shaken at 100 rpm; **a)** pH of AS assays (○), pH of chemical control assays (●) and cell counts of *At. ferrooxidans* assays (x); **b)** solubilized ions in the leachate of *At. ferrooxidans*, iron (◊), copper (□) and sulfate (○); **c)** solubilized ions in the leachate of the sterile control, iron (♦), copper (■) and sulfate (●).

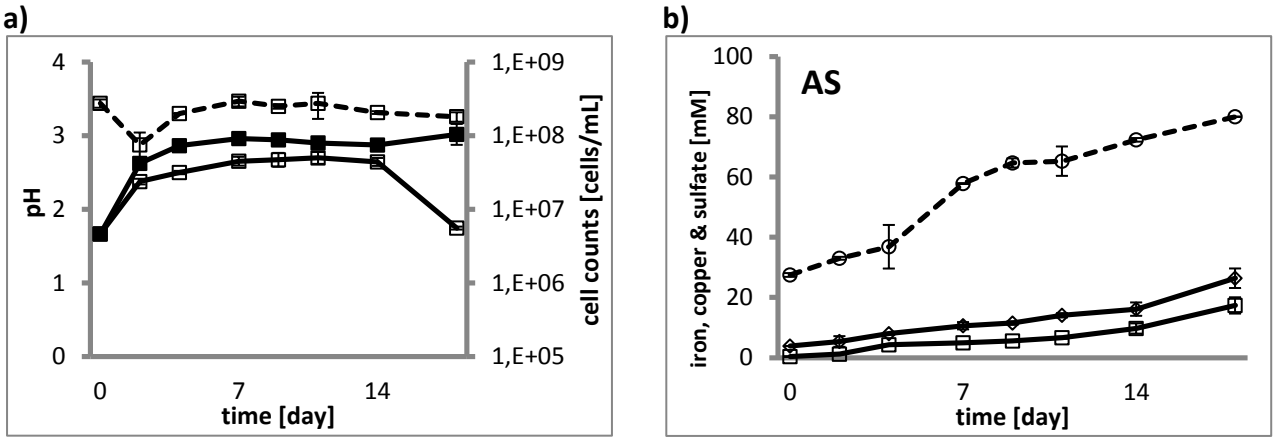

Fig. 74: Bioleaching of chalcopyrite by the moderate thermophilic enrichment AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 45°C and shaken at 100 rpm; **a)** pH of AS assays (○), pH of chemical control assays (●) and cell counts of AS assays (x); **b)** solubilized ions in the leachate of AS, iron (◊), copper (□) and sulfate (○); **c)** solubilized ions in the leachate of the sterile control, iron (♦), copper (■) and sulfate (●).

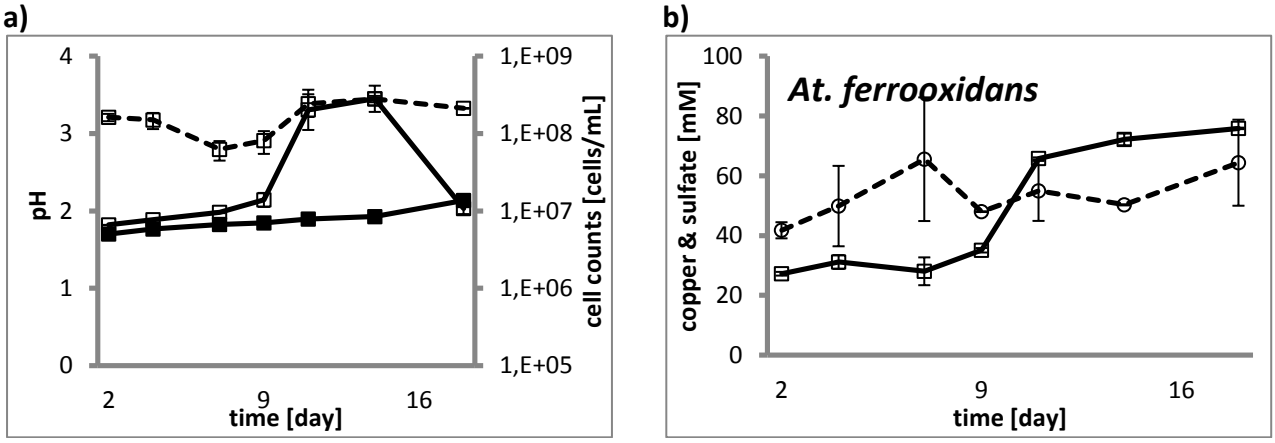

Fig. 75: Bioleaching of covellite by *At. ferrooxidans*. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % covellite (62-200 µm) incubated at 28°C and shaken at 100 rpm; **a)** pH of AS assays (○), pH of chemical control assays (●) and cell counts of *At. ferrooxidans* assays (x); **b)** solubilized ions in the leachate of *At. ferrooxidans*, iron (◊), copper (□) and sulfate (○); **c)** solubilized ions in the leachate of the sterile control, iron (♦), copper (■) and sulfate (●).

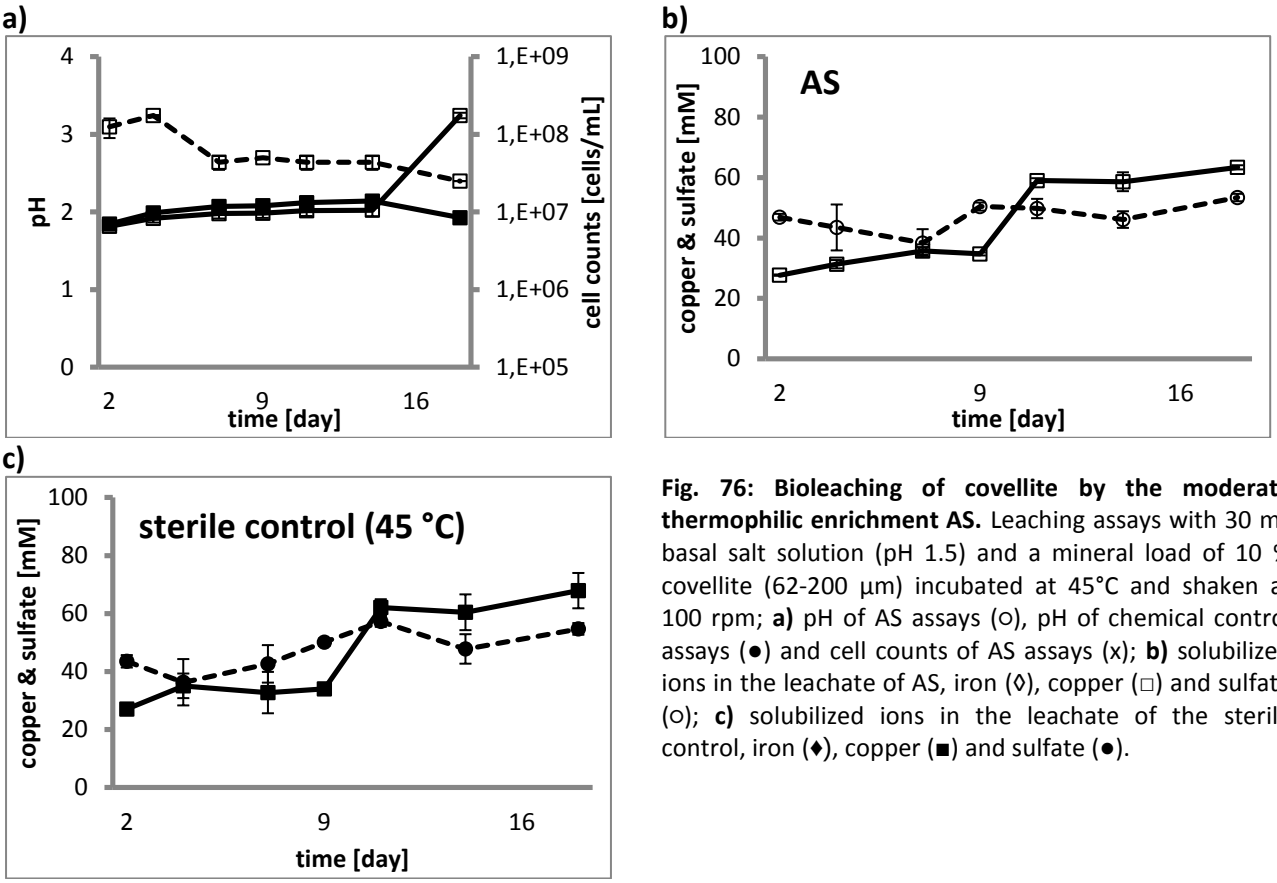

Fig. 76: Bioleaching of covellite by the moderate thermophilic enrichment AS. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of 10 % covellite (62-200 µm) incubated at 45°C and shaken at 100 rpm; **a)** pH of AS assays (○), pH of chemical control assays (●) and cell counts of AS assays (x); **b)** solubilized ions in the leachate of AS, iron (◊), copper (□) and sulfate (○); **c)** solubilized ions in the leachate of the sterile control, iron (♦), copper (■) and sulfate (●).

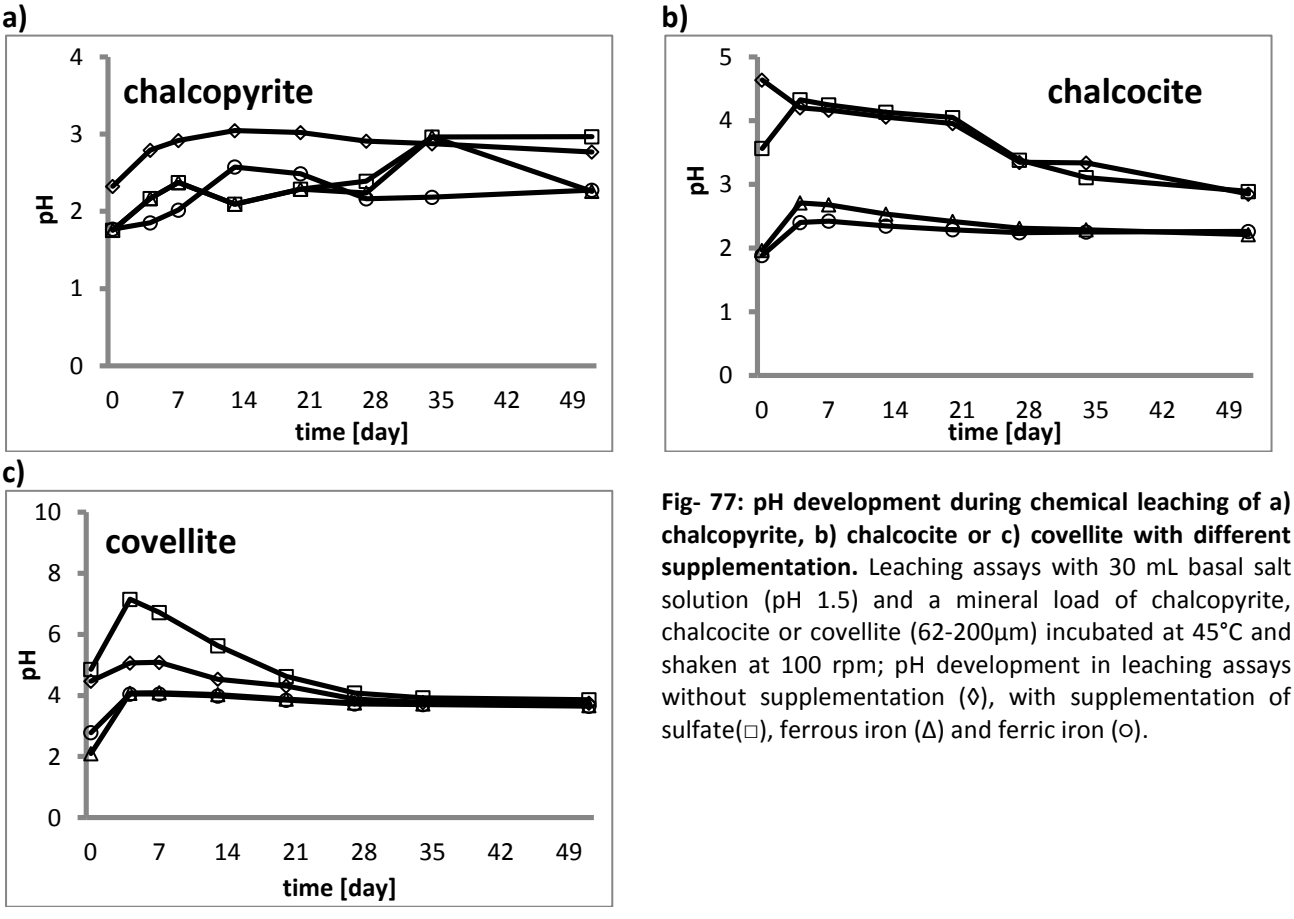

Fig- 77: pH development during chemical leaching of a) chalcopyrite, b) chalcocite or c) covellite with different supplementation. Leaching assays with 30 mL basal salt solution (pH 1.5) and a mineral load of chalcopyrite, chalcocite or covellite (62-200µm) incubated at 45°C and shaken at 100 rpm; pH development in leaching assays without supplementation (◊), with supplementation of sulfate(□), ferrous iron (Δ) and ferric iron (○).

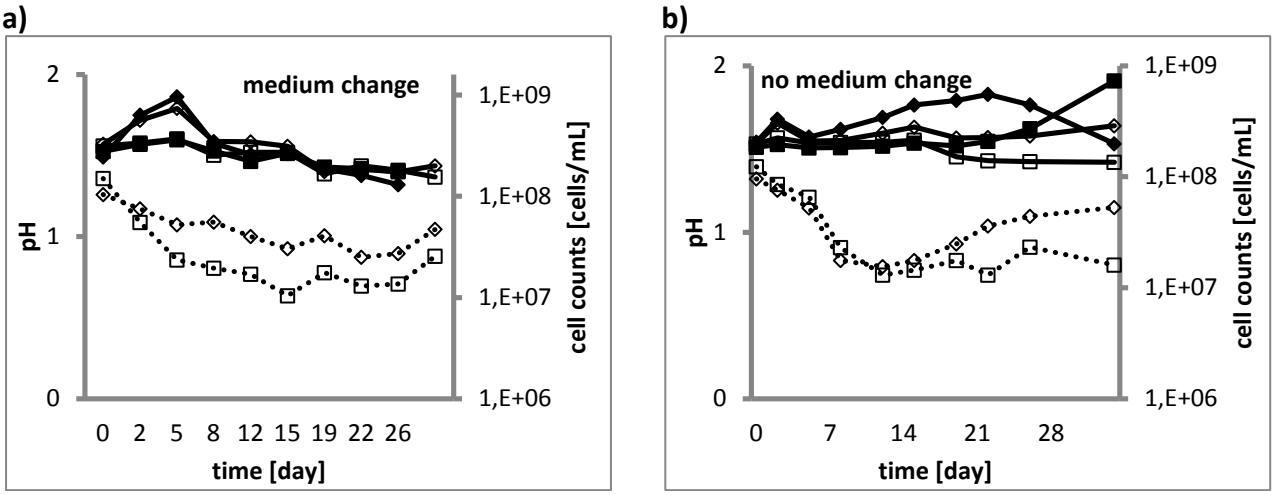

Fig. 78: Copper and iron speciation during bioleaching of chalcopyrite a) with or b) without medium change by the mesophilic enrichment culture AS. Leaching assays with 50 mL basal salt solution (pH 1.5) and a mineral load of 1 % or 10 % chalcopyrite (Siegerland ore, 62-200 µm) incubated at 45 °C and shaken at 100 rpm, basal salt solution was changed each time a sample was taken, cells were centrifuged and transferred with fresh basal salt solution to the batch back; pH AS 10 % (bold line ◊), pH sterile control 10 % (bold line ♦), cell counts AS 10 % (dashed line ◊), pH AS 1 % (bold line □), pH sterile control 1 % (solid line ■) cell counts AS 1% (dashed line □).

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Persönliches

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Berufliche Erfahrung

10/2011 - 09/2015 **wissenschaftliche Mitarbeiterin**
Aquatische Biotechnologie, Biofilm Centre, Universität Duisburg - Essen

- Betreuung und Leitung von Vorlesungen, Praktika und Bachelor/ Masterarbeiten (in Deutscher sowie Englischer Sprache)
- Ausführung und Betreuung von Projekten (Laborarbeit, Kundenbetreuung, Finanzierung, Bestellungen, Berichte, Analyse und Präsentation der Ergebnisse)
- Mikrobiologische und Analytische Laborarbeit unter
- Mikroskopie (EFM, CLSM, AFM)
- Molekularbiologie (PCR, SDS, DGGE, Proteomics)
- Präsentation der Ergebnisse durch Publikationen und Konferenzen

08/2009 - 09/2011 **wissenschaftliche Hilfskraft**
Aquatische Biotechnologie, Biofilm Centre, Universität Duisburg - Essen

- Mikrobiologische und Analytische Laborarbeit

03/2008 - 07/2008 **Werksstudent der Firma Ashland**
am Standort Stora Enso Eilenburg

- selbständige Betreuung einer Down Scale Unit im Prozesswasserkreislauf
- Mikrobiologische und Analytische Laborarbeit
- Analyse und Präsentation der Ergebnisse

08/2002 - 03/2006 **Verkaufshilfe mit Kassentätigkeit**
Kaiser´s Tengelmann

- Warenpräsentation und Warenbeschaffung aus den Lagerbeständen
- Vereinnahmung und Abrechnung der Zahlungsmittel

Studium und Ausbildung

Seit 10/2011	**Promotion** **Aquatische Biotechnologie, Biofilm Centre, Fakultät Chemie,** **Universität Duisburg- Essen** ▪ Thema: Microcalorimetric Investigation on Copper Sulfides
10/2008 - 11/2010	**M.Sc. Water Science** **Universität Duisburg- Essen** ▪ Studienschwerpunkt: Chemie, Mikrobiologie und Analytik ▪ Masterarbeit: Proteomic Analysis of Biofilm Formation in acidophilic Leaching Microorganisms (Note: sehr gut 1,3) ▪ Abschlussnote: gut (2,0)
10/2005 - 07/2008	**B.Sc. Water Science** **Universität Duisburg- Essen** ▪ Studienschwerpunkt: Chemie, Mikrobiologie und Analytik ▪ Bachelorarbeit: Catalase in the Deinking Process of Paper (Note: gut 2,0) ▪ Abschlussnote: befriedigend (2,7)
08/2002 - 06/2005	**Allgemeine Hochschulreife** **Gesamtschule Saarn, Mülheim an der Ruhr**
08/1996 - 06/2002	**Fachoberschulreife** **Realschule an der Mellinghofer Straße, Mülheim an der Ruhr**
08/1992 - 06/1996	**Gemeinschaftsgrundschule** **an der Nordstraße, Mülheim an der Ruhr**

Weiterbildung

2009	**Trinkwasserprobenehmerschulung** IWW Zentrum Wasser, Mülheim an der Ruhr
2010	**Analytisches Praktikum** **Instrumentelle Analytik, Fakultät Chemie, Universität Duisburg- Essen** Kinetics of the Oxidation Process of perfluorinated Compounds in Waste Water Treatment
2010	**Forschungspraktikum** **Aquatische Biotechnologie, Biofilm Centre, Fakultät Chemie,** **Universität Duisburg- Essen** Proteomics of the Biofilm Formation of *Acidithiobacillus ferrooxidans*
2011	**BIOCOR INT Summer School** **University of Portsmouth, UK** Understanding Bioccorosion: Fundamentals & Applications)

Konferenzen

2010	**Biofilm IV** Winchester, UK
2011	**TAM User Meeting** Leipzig
2012	**Innovationsforum Geobiotechnologie** Freiberg
2012	**GeoHannover** Hannover
2013	**TAM User Meeting** Berlin
2013	**International Biohydrometallurgy Symposium** Antofagasta, Chile
2014	**IWA Conference "The perfect slime"** Essen
2015	**TAM User Meeting** Oberhausen

Publications

Vera, M., Janosch, C., Bellenberg, S., Krok, B., Sand, W., Poetsch, A. (2012) New insights into the biofilm lifestyle and metabolism of Acidithiobacillus species from analysis of high throughput proteomic data. Schriftenreihe der Deutschen Gesellschaft für Geowissenschaften 80 87

Krok, B., Schippers, A., Sand, W. (2012) Copper winning by bioleaching of chalcopyrite with mesophilic, moderate thermophilic and extreme thermohilic microorganisms Schriftenreihe der Deutschen Gesellschaft für Geowissenschaften 80 402

Krok, B., Schippers, A., Sand, W. (2013) Copper Recovery by Bioleaching of Chalcopyrite: A Microcalorimetric Approach for the Fast Determination of Bioleaching Activity. Advanced Materials Research 825 322-325

Vera, M., Krok, B., Bellenberg, S., Sand, W. (2013) Shotgun proteomics study of early biofilm formation process of Acidithiobacillus ferrooxidans ATCC 23270 on pyrite. Proteomics 13 1133-1144

Vera, M., Janosch, C., Bellenberg, S., Krok, B., Sand, W., Poetsch, A. (2013) New Insights into the Biofilm Lifestyle and Metabolism of Acidithiobacillus Species from Analysis of High Throughput Proteomic Data. Integration of Science and Industrial Knowledge on Biohydrometallurgy; Guiliani, Demergasso, Quatrini, Remonsellez, Davis-Belmar, Levican, Parada, Barahona, Zale; Trans Tech Publications 2013

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Microcalorimetric Investigation on Copper Sulfide Bioleaching"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im Januar 2016

(Beate Agnes Krok)